AD_____

Award Number: DAMD17-96-1-6179

TITLE: A Cohort Study of the Relationship Between c-erbB-2 and Cyclin D1 Overexpression, p53 Mutation and/or Protein Accumulation, and Risk of Progression From Benign Breast Disease to Breast Cancer; and Creation of a Bank of Benign Breast Tissue

PRINCIPAL INVESTIGATOR: Rita A. Kandel, M.D.

CONTRACTING ORGANIZATION: Mount Sinai Hospital
Toronto, Ontario Canada M5G 1X5

REPORT DATE: October 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	October 2000	Annual (13 Sep 99 – 1 Sep 00)

4. TITLE AND SUBTITLE
A Cohort Study of the Relationship Between c-erbB-2 and Cyclin D1 Overexpression, p53 Mutation and/or Protein Accumulation, and Risk of Progression From Benign Breast Disease to Breast Cancer; and Creation of a Bank of Benign Breast Tissue

5. FUNDING NUMBERS
DAMD17-96-1-6179

6. AUTHOR(S)
Rita A. Kandel, M.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Mount Sinai Hospital
Toronto, Ontario Canada M5G 1X5

E-MAIL:
rkandel@mtsinai.on.ca

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for public release; distribution unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

In a case control study, nested within a cohort of 4,888 women with benign breast disease, we demonstrated that p53 protein accumulation detected by immunohistochemistry was associated with a two-fold increase in risk to develop breast cancer. The purpose of this project is: 1) collect paraffin-embedded benign breast tissue from the remaining 4,336 cohort members who were not part of the case-control study. (2) to enlarge the completed case-control study of p53 with an additional 63 cases (and 5 matched controls per case) and examine the biopsies from these subjects for evidence of c-erbB-2 overexpression and p53 protein accumulation. As well, we will examine the tissue for the presence of p53 mutations in this group and (3) examine whether cyclin D1 amplification and/or protein overexpression is a molecular marker of risk of progression from BBD to breast cancer.

The collection of paraffin blocks is ongoing. We have analyzed all the p53 immunopositive breast tissues and 15 subjects whose breast tissue did not show p53 protein accumulation. p53 sequence changes occurred in 59.2% (16/27) of p53 immunopositive tissues and of these 33% (9/27) were p53 mutations. Four (26.7%) of the immunonegative tissues showed gene alterations of which one was a mutation.

The existing case-control group was examined for cyclin D1 changes. Twelve cases and 29 controls showed cyclin D1 amplification. After adjustment for confounding there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 amplification. Cyclin D1 overexpression was seen in 75 samples of benign breast disease (15 cases and 60 controls). The presence of cyclin D1 overexpression was not associated with increased risk of developing breast cancer (unadjusted odds ratio = 1.07, 95% confidence intervals = 0.56-2.03).

14. SUBJECT TERMS	15. NUMBER OF PAGES
Breast Cancer	71
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

TABLE OF CONTENTS

INTRODUCTION

We have completed a study of the associations between c-erbB-2 protein overexpression and p53 protein accumulation in benign breast tissue and the risk of subsequent breast cancer (1). The study was conducted as a case-control study nested within the cohort of 4888 women in the National Breast Screening Study of Canada (NBSS) who were diagnosed with benign breast disease and underwent active follow-up. Case subjects were the women who subsequently developed breast cancer (ductal carcinoma in situ (DCIS) or invasive carcinoma). Control subjects were matched to each case subject by NBSS study arm, screening center, year of birth, and age at diagnosis of benign breast disease. Histologic sections of benign and cancerous breast tissues were analyzed immunohistochemically. Information on potential confounding factors was obtained by use of a self-administered lifestyle questionnaire completed at the time of enrolment. Accumulation of p53 protein was associated with an increased risk of progression to breast cancer (adjusted odds ratio (OR) = 2.55; 95% confidence interval (CI) = 1.01-6.40), whereas c-erbB-2 protein overexpression was not (adjusted OR = 0.65; 95% CI = 0.27-1.53). The findings for c-erbB-2 and p53 did not differ among stratas defined by menopausal status, allocation within the NBSS, history of breast disease, and whether the benign breast disease was detected at a scheduled screen or between screens. The results were also similar after exclusion of case subjects whose diagnosis of breast cancer occurred within 1 year of their diagnosis of benign breast disease and after exclusion of subjects with DCIS. In summary, p53 protein accumulation, but not c-erbB-2 protein overexpression, appeared to be associated with an increased risk of progression to breast cancer in women with benign breast disease..

The purpose of this project is threefold. Specifically, we are:

(1) collecting paraffin-embedded benign breast tissue from the remaining 4,336 cohort members who are not part of the case-control study. This will establish a bank of paraffin-embedded tissue for a cohort of women on whom there is extensive documentation of risk factor information. With further follow-up of the cohort, it will be possible to enlarge the above described case-control study, and to undertake additional studies of newly identified molecular markers of risk of progression to breast cancer.

(2) enlarging our recently completed case-control study of p53 with an additional 63 cases (and 5 matched controls per case) which were identified as a result of a linkage of the NBSS database to the Canadian Cancer Database. We propose to examine biopsies from these subjects for evidence of c-erbB-2 overexpression and p53 protein accumulation. Addition of these cases and controls to the previous study will increase its statistical power. As well, we will examine the tissue for the presence of p53 mutations in this group of women.

(3) examining whether cyclin D1 amplification and/or protein overexpression determined immmunohistochemically is a molecular marker of risk of progression from BBD to breast cancer in the enlarged case-control study. We hypothesize that cyclin D1 overexpression in benign breast disease is associated with increased risk of progression to breast cancer.

Epithelial cancers appear to be the result of an accumulation of multiple genetic events (2-4). Multiple molecular markers are being examined, since progression to cancer probably results from the accumulation of several genetic events (5). To date, in relation to breast cancer, cyclin D1 and c-erbB-2 overexpression, and p53 mutations are amongst the more frequent genetic changes detected. While cyclin D1, c-erbB-2, and p53 appear to play an important part in mammary carcinogenesis, their precise role in this process is unclear. For example, it is unclear whether they are involved in the initiation of transformation or at a subsequent stage, or whether they are just indicators of increased risk of developing breast cancer as they may be markers of genetic instability. Further study will be required to determine their contributions.

BODY

(1) STUDY DESIGN:

Our study uses paraffin-embedded breast tissues, which have been obtained from the cohort of women enrolled within the National Breast Screening Study (NBSS) and who received a diagnosis of benign breast disease during the active follow-up phase of the study. In the ensuing paragraphs, we describe the NBSS first, and then present details of the collection of paraffin blocks and subsequent investigations.

***(a) The National Breast Screening Study*:** The NBSS is a multi-center randomized controlled trial of screening for breast cancer in Canadian women aged 40 to 59 at recruitment (6,7). The study involves 89,835 women who were recruited at 15 screening centers across Canada. Recruitment commenced in 1980 and ended in 1985. Women were eligible to participate in the study if they had no history of breast cancer, were not currently pregnant, and had not had a mammogram in the preceding 12 months.

Women aged 40-49 years were randomized either to have annual mammography plus physical examination, or to have initial physical examination only, and women in both the intervention and the control group were taught breast self-examination. Randomization in the 50-59 year age group was either to annual mammography plus physical examination, or to annual physical examination alone (women in this arm of the 50-59 year age group were also taught breast self-examination).

***(i) Diagnosis of BBD and breast cancer in the NBSS*:** At each visit, study participants had a physical examination. For those who were randomized to the intervention group, physical examination was followed by mammography, the films from which were read by a study radiologist who was unaware of the physical examination results. If the examiner or the radiologist reported an abnormality requiring further assessment, a referral was made to a review clinic where the participant was seen by a study surgeon. If, on review, a recommendation was made for biopsy, this recommendation was conveyed to the participant's family physician, and the participant was contacted and asked to visit her family physician for further management.

Women in both control groups were referred for mammography if either they or their primary care physician discovered an abnormality for which referral was warranted. Staff in each screening center identified the pathology laboratory in which biopsies were examined, and they obtained slides or blocks for review by a locally designated reference pathologist. Results of the histological review of the biopsies were forwarded to the coordinating center.

***(ii) Follow-up in the NBSS*:** Active follow-up continued until 1988. During this phase of the NBSS (when the study participants underwent the screening schedule corresponding to their allocation, as described in (a) above), there was in each study center a coordinator (usually a nurse) who had experience in clinic or study management. The coordinators were responsible for ascertaining whether the

recommended diagnostic procedures had been carried out and for collecting reports of the surgical and pathological procedures from the institutions where they had been performed. Procedures performed independently of the screening process were identified through annual questionnaires sent to study subjects, and reports of these procedures were then obtained from the relevant institutions. Study records for women who moved out of their original area were transferred to the center nearest their new residence. Following completion of their screening schedule, direct follow-up stopped for those with no diagnosis of breast cancer. However, until 1988-1990 (depending upon the province) information about new diagnoses of breast cancer was obtained by linkage with the provincial cancer registries (cancer is registered in each province in Canada, and, for Ontario at least, registration is essentially complete (8)). Subsequently, new diagnoses of cancer will be ascertained by linkage to the Canadian Cancer Database, which is operated by the Canadian Center for Health Information at Statistics Canada, and consists of registration data reported annually by the provincial registries. A linkage yielding incidence data to the end of 1993 was completed recently, and we propose that another linkage take place in mid 2000 to yield a further four to five years of follow-up data.

(b) Description of the cohort: The immunohistochemical and molecular investigation currently underway is being undertaken within the cohort of 4,888 women within the NBSS who received a histopathologic diagnosis of BBD during the active follow-up phase of the NBSS. In order to reduce costs substantially while having relatively little impact on the precision of the estimates of association (9), the study is being conducted as a case-control study nested within this cohort. Cases are women who subsequently developed breast cancer, while controls are women who had not developed breast cancer by the date of diagnosis of the corresponding case. Five controls were selected for each case, and they are matched to the corresponding case on study arm within the NBSS, screening center, year of birth, and age at diagnosis of BBD.

(i) Case definition: Cases are women with a history of BBD detected during the course of the NBSS who subsequently developed breast cancer. By this definition, 92 cases were identified by previous linkages. We collected the benign tissue from 74 cases. As described below, as a result of the linkage to the 1993 database, we have identified 63 additional cases.

(ii) Definition of controls: Controls are women who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case (they will, of course, have a diagnosis of BBD). Since there are no estimates of the likely magnitude of the effects of interest on risk of progression from BBD to breast cancer, we select 5 controls for each case in order to maximize statistical power. Controls are matched to cases on study arm within the NBSS, screening center, year of birth, and age at diagnosis of BBD (and implicitly on the interval between diagnosis of BBD and the date of diagnosis of breast cancer in the corresponding case). These matching criteria are chosen either because the factors of interest are related to breast cancer risk (age, and possibly age at diagnosis of BBD) or because they are related to the risk of disease detection (allocation and screening centre). It is also conceivable that at least some of these factors are related to the exposures of interest. However, it should be noted that

little is known about the "epidemiological" correlates of cyclin D1 and c-erbB-2 overexpression, and p53 protein accumulation. Additionally, the implicit matching on duration of follow-up (as well as age) means that the controls have had the same opportunity (at least, in terms of the elapse of time) to develop breast cancer as the cases.

(iii) ***Questionnaires***: At the time of their enrolment in the NBSS, all participants completed a questionnaire which sought identifying information, as well as data on factors such as demographic characteristics, family history of breast cancer, menstrual and reproductive history, use of oral contraceptives and replacement estrogens, and cigarette smoking. Additionally, approximately two-thirds of the 89,835 women enrolled in the NBSS completed self-administered diet history questionnaires. The dietary questionnaire was introduced in 1982, at which time some women had already been enrolled in the study (and were not seen again at the screening centers). The diet history contained questions on the frequency of consumption and usual portion size of 86 food items, and also had an open-ended section for describing other food items normally eaten. Photographs of various portion sizes were included in the questionnaire to assist participants to quantify intake. The questionnaire also included questions on current and past height and weight, and on consumption of beer, wine, and spirits. A comparison between the self-administered questionnaire and a full interviewer-administered questionnaire which has been subjected to both validity and reliability testing (10) and used in a number of epidemiologic studies (11) revealed that the two methods give similar results for the major macronutrients, dietary fiber, and vitamin C (12).

(iv) ***Statistical power:*** This was calculated according to that described by Breslow and Day (13).

(2) CONDUCT OF THE STUDY:

(a) ***Coding, data entry, and processing***: The lifestyle information is available on the computerized NBSS database. The standard procedures of the Cancer Epidemiology Unit for quality control are used for coding and data entry.

(b) ***Collection of paraffin-embedded breast material***: For the completed case-control study of p53 and c-erbB-2 protein changes in benign breast disease, we created a database consisting of identifying information, plus details of the location and accession number of the 552 paraffin blocks. This information was used to generate lists for each hospital of the study participants for whom we wished to obtain paraffin blocks. We then wrote to the pathologist-in-chief at the hospital seeking the blocks.

This same approach was used to expand the existing tissue bank. The database was updated to include all 4,888 subjects with a diagnosis of BBD in the NBSS. We are currently attempting to collect the blocks of the remaining 4,336 (4,888-552) women.

(c) ***Histopathological Review***: Sections from blocks received for the expanded nested case-control study will be reviewed and classified by Dr. Kandel and a collaborator, Dr.

W. Hartwick, according to the criteria developed by Page (14), and as described in the consensus conference for DCIS (15). Briefly, in benign lesions, the presence or absence of epithelial proliferation is determined, and when epithelial proliferation is present, the lesions were classified further according to the presence or absence of cytological atypia. The cancers are classified by histological type.

(d) Experimental methods: In this section we describe the methodology that was used to evaluate cyclin D1 gene amplification and protein overexpression in the nested cohort of women that was used to assess p53 and c-erbB-2. For completeness we also present details of the immunohistochemical staining for c-erbB-2 and p53 as well as the molecular analysis of p53.

(i) Cyclin D1 in Breast Tissue:

Cyclin D1 immunohistochemistry

Since we do not have access to frozen tissue, immunohistochemical staining was used to detect cyclin D1 overexpression. The antibody that we selected works on paraffin-embedded tissue. Immunohistochemical staining allows cellular localization of the immunoreactivity, so it was possible to ensure that the cyclin expression was occurring in breast epithelial cells. In addition, this approach allowed us to determine whether the immunoreactivity was present in the histopathology considered to be associated with increased malignant potential. Breast cancers were stained in order to determine whether the expression present in the benign breast disease was maintained in the malignant lesion, or was present in the breast cancer only.

Tissue sections were placed on 2% aminopropyltriethoxysilane (Sigma)-coated slides and deparaffinized. The tissue underwent antigen retrieval and incubated overnight at 4°C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY) as described previously (16). After washing, the sections were incubated with biotinylated antimouse immunoglobulin G (dilution 1:200; Vector Laboratories), followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories). Immunoreactivity was visualized with 3,3'-diaminobenzidine (Vector), and the sections were counterstained briefly with hematoxylin. T47D cells embedded in paraffin served as the positive control (17). The negative control consisted of replacing the primary antibody with Tris-buffered saline or nonimmune mouse serum (DAKO, Carpinteria, CA). Distinct nuclear staining in greater than 1% of epithelial cells indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative.

Cyclin D1 amplification

Five um thick sections were cut, briefly stained with hematoxylin. The epithelium in the tissue which showed cyclin D1 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections showing no cyclin D1 protein accumulation immunohistochemically underwent random microdissection of epithelium. DNA was extracted by incubating the microdissected tissue in buffer (50 mM Tris-HCl, pH 8.5, 1 mM ethylenediamine tetracetic acid, 0.5% Tween 20) containing 0.5 mg/ml of

proteinase K (Sigma Chemical Co, St. Louis, MO) at 50°C for 48 hours. The proteinase K was then inactivated by boiling at 95°C for 15 minutes.

Semiquantitative differential polymerase chain reaction (PCR) was used to determine the presence of cyclin D1 gene amplification. As fragmented genomic DNA (<200 bp) may influence the results of differential PCR, γ-interferon (γ-IFN) was analyzed in a multiplex PCR reaction in order to indirectly assess DNA quality first (18,19). Two sets of primers, specific for different exons of γ-IFN gene and which generate PCR products of 150 and 82 bp (γ-IFN 150 and γ-IFN 82) were co-amplified in the same reaction tube as described previously (19). If the γ-IFN82/γ-IFN150 ratio of the PCR products was 3 or less, the tissue was considered suitable for further analysis. For such cases, aliquots of the proteinase K digested tissue were then examined for cyclin D1 amplification using PCR. Both dopamine receptor (DR) and cyclin D1 were co-amplified in the same reaction tube. DR was chosen as it is present on the same chromosome as cyclin D1 (20). If the tissue had chromosomal duplication it would simulate amplification and would be a false positive. To prevent this we selected a gene on the same chromosome yet of sufficient distance from cyclin D1 that it was unlikely to be part of an amplified amplicon. Included in each run was DNA extracted from two paraffin embedded cell lines; MDA-MB-231 which shows no gene amplification and ZR-75-1 which has cyclin D1 amplification (21,22). Briefly, 1 l of the digest was mixed with 14 l of PCR working solution containing 10mM Tris Hcl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 M of eachdNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems Inc., Branchburg, NJ) and 1 M of each primer. The samples underwent 30 cycles of amplification in an automated thermocycler (DNA Thermal Cycler, Perkin Elmer, Branchburg, NJ). Each cycle consisted of 1.2 minutes of denaturation at 94°C, 1 minute of annealing at 55°C, and 1 minute of elongation at 72°C. The PCR products were separated by electrophoresis on a 12% polyacrylamide gel at 200V for 2 hours and visualized following ethidium bromide staining. Each tissue was analyzed at least twice in separate polymerase chain reactions. Each sample showing amplification of cyclin D1 was then repeated one additional time. Concurrently, DNA extracted from the subject's breast stromal tissue, which should show no amplification, was also analyzed as a control. Direct sequencing of selected PCR products using the sense primer and the Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA) confirmed the specificity of the primers (23).

To determine whether there was cyclin D1 gene amplification, the ratio of the cyclin D1 PCR product to the DR PCR product was derived from photographic negatives of ethidium bromide stained gels, which were quantified by laser densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA). There were at least two gels per sample and each gel was scanned three times. A mean ratio of cyclin D1 to DR was determined and a ratio of greater than 0.88 was considered indicative of gene amplification. This value was determined by calculating two standard deviations from the average of the ratios (n=93) obtained from the negative control cell line that had no gene amplification.

(ii) Assessment of p53 accumulation, c-erbB-2, and cyclin D1 overexpression in breast tissue

The subjects newly identified by the recently completed linkage will be used to expand the previously published study (1). The paraffin blocks from these individuals will undergo immunostaining for p53, c-erbB-2, and cyclin D1 using standard immunohistochemical methods as described either above (for cyclin D1) or previously (p53 and c-erbB-2) (1).

(iii) Methodology to detect p53 mutations in breast tissue

As well we are examining whether p53 mutations occur in the tissues from the subjects in this study (1) using PCR-SSCP and manual sequencing when indicated.

Five m sections are cut from the paraffin blocks dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue that shows p53 immunoreactivity is microdissected out and placed in a microfuge tube. The tissue is digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hrs at 55°C (23). The proteinase K is inactivated (95°C for 15 min).

An aliquot of the digest is amplified using PCR, [^{33}P]-dATP and exon-specific primers. An aliquot of the reaction product is separated on an 8% non-denaturing polyacrylamide gel and the gel is processed for autoradiography (24,25). Potential mutations are detected by shifts in band mobility. If no band shifts are detected in these samples, the tissue are considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis will be repeated. If the two PCR-SSCP analyses generate different band shifts, another section will be cut, microdissected and processed for PCR-SSCP analysis as described above.

The abnormally shifted band is excised from SSCP gels and the DNA eluted into water. The DNA is reamplified by PCR using the same primers and the product run on a 2% agarose gel. The band is extracted using QIAquick gel extraction kit (Qiagen Inc, Mississauga, ON). The purified DNA is manually sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product will be resequenced using the antisense primer. Negative controls will be included in each analysis. Gene alterations will be compared to those listed for breast cancer in a p53 database (http://www.iarc.fr/p53).

(e) Statistical analysis: Essentially, the statistical analysis involves comparison of the frequency (either singly or in combination) of cyclin D1, c-erbB-2 overexpression and p53 protein accumulation and mutations in the cases and controls, using conditional logistic regression with multiple controls per case (9). The association between these genetic changes and factors which are thought to be involved in the etiology of BBD and breast cancer (e.g., reproductive, menstrual, and dietary factors, as well as BBD histology) are examined, as well the association of the latter variables with risk of progression to breast cancer.

Further analyses will be directed towards within-individual comparisons of cyclin D1 and c-erbB-2 overexpression and p53 in BBD and breast cancer. One possible interpretation of any changes that are found to be common to both conditions will be that they contribute to the development of breast cancer rather than arise as a consequence of it.

RESULTS

Technical Objective 1: Obtaining blocks from remaining 4888 cohort members

We have updated the database with respect to identifying details of the remaining individuals in the cohort with benign breast disease.

We have contacted a total of 253 hospitals. The number has changed from our grant proposal because of the ongoing hospital mergers that are occurring in Canada. We are in the process of accessioning the paraffin blocks received to date. 157 hospitals/ laboratories have sent 2242 blocks out of 2635 requested (85%). We are in contact with 17 hospitals of which have agreed to send blocks (52 blocks) but have not done so yet. The remaining 14 hospitals are in discussion with us and have indicated that they may be willing to send the blocks that we have requested (412 blocks). Fifty-four hospitals (1153 blocks) have replied and informed us that the blocks requested have been discarded and 25 hospitals had sent the blocks to other locations. Repeated follow-up phone calls are being made to the lab director or their designate for the outstanding 17 hospitals.

Technical Objective 2: Assessing role of cyclin D1 in progression of BBD to breast cancer

Cyclin D1 gene levels were assessed in tissues from 356 subjects. Cyclin D1 immunostaining of tissue sections from 383 blocks of benign breast disease was also done.

Twelve cases and 29 controls showed cyclin D1 amplification. Gene amplification values ranged from 0.89 to 1.27. Previous studies have shown that the breast cell line ZR-75-1, which was used as positive control, had approximately a three-fold amplification (26,27) and this cell line using our methodology had on average an amplification ratio of 1.26 ± 0.96. This suggests that when cyclin D1 in the breast tissues was amplified it had at most three-fold amplification. After adjustment for confounding there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 amplification.

Cyclin D1 overexpression was seen in 75 samples of benign breast disease. Fifteen of the immunopositive tissues were cases and 60 were controls. When the analysis was completed the presence of cyclin D1 overexpression was not associated

with increased risk of developing breast cancer (unadjusted odds ratio = 1.07, 95% confidence intervals = 0.56-2.03).

This objective has been accomplished. The results have been analyzed and a manuscript detailing our findings submitted to European Journal of Cancer (see appendix).

Technical Objective 3: Cyclin D1, p53 and cerbB-2 immunostaining, cyclin D gene amplification and p53 molecular analysis of additional cases and controls identified in 2000 linkage

The tasks in this objective are dependant on the extension of the ongoing project by the addition of more cases and their controls identified as a result of longer follow-up. The cases are identified by the linkage of the NBSS database to the Canadian Cancer Database and this was delayed at Statistics Canada. Once the linkage was done the breast cancer diagnoses had to be verified and this took more time than anticipated for technical reasons. However, all this has been completed and an additional 63 cases were identified and the controls were selected. As a result of the delay in the linkage the work to accomplish tasks 6 to 9 are ongoing. These should be completed by the end of the extended grant period.

We have developed the methods to detect p53 mutations in DNA extracted from paraffin embedded tissue (task 7). We have analyzed all the p53 immunopositive breast tissues and 15 subjects whose breast tissue did not show p53 protein accumulation. p53 sequence changes occurred overall in 59.2% (16/27) of p53 immunopositive tissues. p53 mutations occurred in 33% (9/27) of immunopositive case. Four (26.7%0 of the immunonegative tissues showed gene alterations of which one was a mutation. The results of this work have been published in the International Journal of Cancer.

Technical Objective 4: Linkage to the National Cancer Incidence Reporting System in calendar year 2000

This objective entails preparing a file for a second linkage to obtain patient clinical follow-up to the year 1997-98. The file was prepared and transferred to Statistics Canada. They are currently doing the linkage and should identify additional cases.

KEY RESEARCH ACCOMPLISHMENTS:

- creation of a tissue bank of benign breast tissue
- demonstrated that cyclin D1 protein overexpression and gene amplification occurs in normal and benign breast tissue
- demonstrated that cyclin D1 protein overexpression is not associated with increased breast cancer risk
- demonstrated that cyclin D1 gene amplification is not associated with increased breast cancer risk
- demonstrated that p53 mutations and gene changes occur in normal and benign breast tissue
- expansion of cohort study which should refine the role of p53 protein accumulation as a marker of increased breast cancer risk

REPORTABLE OUTCOMES:

A) The following publications, manuscripts, and abstracts have resulted from the work supported by this grant.

1. Zhu XL, Rohan T, Hartwick W, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. Mod Pathol 11: 1082-1088, 1998.

2. Kandel RA, Li, S-Q, Ozcelik H, Rohan T. p53 protein accumulation and mutations in normal and benign breast tissue. Int J Cancer 87:73-78, 2000.

3. Pollett A, Bedard YC, Li S-Q, Rohan T, Kandel RA. Correlation of p53 mutations in Thin Prep Processed Fine needle aspirates with surgically resected breast cancers. Mod Pathol, in press Nov, 2000.

4. Kandel RA, Zhu XL, Li S-Q, Rohan T. Cyclin D1 overexpression and gene amplification in benign breast tissue and breast cancer risk. submitted Eur J Cancer, 2000.

5. Duffy SW, Rohan TE, Kandel R. Misclassification in a matched case-control study with variable matching ratio. Submitted to Statistics in Medicine, 2000.

6 Rohan T, Zhu X-L, Kandel R. Cyclin D1 in benign breast disease and risk of breast cancer. Proceedings of the American Association for Cancer Research, Philadelphia, PA, April, 1999.

7. Pollett AF, Bedard YC, Rohan T, Kandel RA. Detection of p53 mutations in ThinPrep® processed fine needle aspirates of breast carcinoma. American Association of Cytopathology, Acta Cytol 43: 922, 1999.

8. Kandel RA, Li S-Q, Rohan T. Cyclin D1 gene amplification in benign breast disease and risk of breast cancer. Proceedings of the American Association for Cancer Research, San Francisco, April, 2000.

B) In addition we received another grant from the US Army Medical Research and Materiel Command (Are p53 mutations associated with increased risk of developing breast cancer? A molecular epidemiological study. #BC980784) that will allow the continuation of this project. This grant focuses on determining whether p53 gene changes are associated with increased risk of developing breast cancer in the entire cohort.

C) With the support of this grant we have been able to establish a tissue bank of paraffin-embedded normal or benign breast tissue.

CONCLUSIONS

As the collection of blocks and the expansion of our case-control study are ongoing, the conclusions that can be drawn are limited. Our recently published case-control study involving the use of benign breast tissue from individuals enrolled in the NBBS has been called a "paradigm for future studies of additional biomarkers that may identify women with high risk benign breast disease" in a recent editorial about our studies (28). This supports the approach that we are using to identify biomarkers of increased breast cancer risk and we are continuing to collect the paraffin blocks of the benign breast tissue to be able to do these types of studies.

We have shown that cyclin D1 protein overexpression occurs in normal and benign breast tissue (23). We found that cyclin D1 gene amplification and protein overexpression as detected immunohistochemically were not associated with increased risk of developing breast cancer in the group of individuals studied to date (see appendix).

REFERENCES

1. Rohan TE, Hartwick W, Miller AB, Kandel RA. Immunohistochemical detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. J Natl Cancer Inst 1998;90:1262-1269.
2. Vogelstein B, Kinzler KW. The multistep nature of cancer. Trends Genet 1993;9:138-141.
3. Kinzler KW, Vogelstein B. Gatekeepers and caretakers. Nature 1997;386:761-763.
4. Ingvarsson S. Molecular genetics of breast cancer progression. Cancer Biol 1999;9:277-288.
5. Beckmann MW, Niederacher D, Schnurch H-G, Gusterson BA, Bender HG. Multistep carcinogenesis of breast cancer and tumour heterogeneity. J Mol Medicine 1997;75:429-439.
6. Miller AB, Howe GR, Wall C. The national study of breast cancer screening: protocol for a Canadian randomized controlled trial of screening for breast cancer in women. Clin Invest Med 1981;4:227-258.
7. Miller AB, Baines CJ, To T, Wall C. Canadian National Breast Screening Study. I. Breast cancer detection and death rates among women aged 40 to 49 years. II. Breast cancer detection and death rates among women aged 50 to 59 years. CMAJ 1992; 147:1459-1488.
8. Robles SC, Marrett LD, Clarke EA, et al. An application of capture-recapture methods to the estimation of completeness of cancer registration. J Clin Epidemiol 1988; 41:495-501.
9. Rothman KJ. Modern epidemiology. Boston: Little, Brown and Co., 1986.
10. Jain MG, Howe GR, Johnson KC, Miller AB. Evaluation of a diet history questionnaire for epidemiologic studies. Am J Epidemiol 1980;111: 212-219.
11. Morgan RW, Jain M, Miller AB, Choi NW, Matthews V, Munan L, Burch JD, Feather J, Howe GR, Kelly A. A comparison of dietary methods in epidemiologic studies. Am J Epidemiol 1978; 107:488-498.
12. Jain MG, Harrison L, Howe GR, Miller AB. Evaluation of a self-administered dietary questionnaire for epidemiologic studies. Am J Clin Nutr 1982;36:931-935.
13. Breslow NE, Day NE. Statistical methods in cancer research. The design and analysis of cohort studies. Lyon: IARC, 1987, 297-300.
14. Page DL, Anderson TJ. Diagnostic Histopathology of the Breast. New York: Churchill Livingstone, 1987.
15. Schwartz GF, Lagios MD, Carter D, Connolly J, Ellis IO, Eusebi V. et al. Consensus conference on the classification of ductal carcinoma in situ. Hum Pathol 1997;28:1221-1224.
16.
17. Bartkova J, Lukas J, Müller H, Lützhøft D, Strauss M, Bartek J. Cyclin D1 protein expression and function in human breast cancer. Int J Cancer 1994;57:353-361.
18. Frye RA, Benz CC, Liu E. Detection of amplified oncogenes by differential polymerase chain reaction. Oncogene 1989;4:1153-1157.
19. Neubauer A, Neubauer B, He M, Effert P, Inglehart D, Frye RA, Liu E. Analysis of gene amplification in archival tissue by differential polymerase chain reaction. Oncogene 1992;7:1019-1025.

20. Grandy DK, Marchionni MA, Makam H, Stofko RE, Alfano M, Frothingham L, Fischer JB, Burke-Howie KJ, Bunzow JR, Server AC, Civelli O. Cloning of the cDNA and gene for a human D_2 dopamine receptor. Proc Natl Acad Sci USA 1989;86:9762-9766.
21. Buckley MF, Sweeney KJE, Hamilton JA, Sini RL, Manning DL, Nicholson RI deFazio A, Watts CK, Musgrove EA, Sutherland RL. Expression and amplification of cyclin genes in human breast cancer. Oncogene 1993;8:2127-2133.
22. Bartkova J, Lukas J, Strauss M, Bartek J. Cell cycle related variation and tissue restricted expression of human cyclin D1 protein. J Pathol 1994;172:237-245.
23. Zhu XL, Hartwick W, Rohan T, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. Mod Pathol 1998;11:1082-1088.
24. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989;5:874-879.
25. Murakami Y, Hayashi K, Sekiya T. Detection of aberrations of the p53 alleles and the gene transcript in human tumor cell lines by single-strand conformation polymorphism analysis. Cancer 1991;51:3356-3361.
26. Lammie GA, Fantl V, Smith R, Schurring E, Brookes S, Michalides R, Dickson C, Arnold A, Peters G. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. Oncogene 1991;6:439-444.
27. Schuuring E, Verhoeven E, Mooi WJ, Michalides RJAM. Identification and cloning of two overexpresed genes, U21B31/PRAD1 and EMS1, within the amplified chromosome 11q13 region in human carcinomas. Oncogene 1992; 7:355-361.
28. Allred DC, Hilsenbeck SG. Editorial. Biomarkers in benign breast disease: risk factors for breast cancer. J Natl Cancer Inst 1998;90:1247-1248.

APPENDIX

Int. J. Cancer: 87, 73–78 (2000)

Publication of the International Union Against Cancer

p53 PROTEIN ACCUMULATION AND MUTATIONS IN NORMAL AND BENIGN BREAST TISSUE

Rita KANDEL[1]*, Shu-Qiu LI[1], Hilmi OZCELIK[1] and Tom ROHAN[2]

[1]*Department of Pathology and Laboratory Medicine and Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada*

[2]*Department of Public Health Sciences, University of Toronto, Toronto, Ontario, Canada*

Mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer, and there are several reports suggesting that changes in p53 may contribute to the pathogenesis of this disease. In a previous case-control study, we demonstrated that p53 protein accumulation detected by immunohistochemistry in normal or benign breast tissue was associated with a 2.5-fold increase in the risk of subsequent breast cancer. In this study, we investigated whether p53 gene mutations were present in the 29 p53 immunopositive normal or benign breast tissue samples and in 15 p53 immunonegative normal or benign breast tissue samples selected randomly from the original study. DNA was extracted from paraffin sections and underwent PCR-SSCP analysis for exons 4 to 10. PCR products that showed abnormal mobility were excised and sequenced. Sixteen (59.2%) of the 27 immunopositive breast tissue samples and 4 (26.7%) of the 15 immunonegative samples had p53 sequence changes. There was no obvious association between the occurrence of these alterations and any specific histopathologic features. Ten cases showed p53 mutations, and they were all missense base substitutions of the transition type. Thirteen other gene changes occurred in 11 breast tissue samples and consisted of 8 silent (no amino acid change), 4 intronic alterations, and 1 indeterminate alteration. One individual had both a mutation and a silent change. In summary, p53 gene alterations can occur in normal or benign breast tissue, but resolution of their role in the pathogenesis of breast cancer will require long-term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations as well as functional assays to determine their significance. *Int. J. Cancer* 87:73–78, 2000.

Carcinogenesis is a complex multistep process that arises from the accumulation of critical genetic changes (Shackney and Shankey, 1997). The molecular changes leading to the development of breast cancer are not well characterized. However, mutations in the p53 gene are amongst the most common molecular changes detected in breast cancer (Phillips *et al.*, 1999) and several clinical and experimental studies have suggested that changes in p53 may contribute to the pathogenesis of this disease.

In experimental studies, p53 mutations occur in the preneoplastic stage of mouse mammary tumour development (Jerry *et al.*, 1993). It has been shown that transgenic mice expressing a mutant p53 172^{R-H} minigene that had been targeted to the mammary gland developed chemically induced breast cancer with shorter latency periods and greater tumour burden than did their nontransgenic littermates (Li *et al.*, 1998). Gao *et al.* (1996) have shown that ablation of p53 function by a dominant negative p53 mutant can result in immortalization of normal human mammary epithelial cells. However, not all dominant negative mutants induce immortalization (Gao *et al.*, 1997), suggesting that the contribution of mutant p53 to the development of cancer is complex.

In clinical studies, p53 mutations and/or p53 protein accumulation have been detected in intraductal carcinomas (Done *et al.*, 1998; Lisboa *et al.*, 1998; Phillips *et al.*, 1999). p53 protein accumulation has also been demonstrated immunohistochemically in the benign breast tissue of patients with the Li-Fraumeni syndrome (Thor *et al.*, 1992) and in benign tissue adjacent to breast cancer in women with a cancer syndrome distinct from Li-Fraumeni syndrome (Barnes *et al.*, 1992). Several reports have also shown p53 mutations and/or positive immunostaining for p53 in sporadic forms of benign breast disease (Millikan *et al.*, 1995; Schmitt *et al.*, 1995; Younes *et al.*, 1995; Lisboa *et al.*, 1997; Rohan *et al.*, 1998). Collectively, these findings suggest that p53 changes can occur prior to the development of breast cancer. This is in keeping with observations by others that p53 alterations can occur in putative precursor lesions of other cancers and in normal tissues. For example, p53 mutations have been detected in Barrett's esophagus (Campomenosi *et al.*, 1996), and mutations in codons 247 and 248 have been detected in normal skin and have been shown to be associated with increased risk of developing basal cell carcinoma (Ouhtit *et al.*, 1998).

In a previous study (Rohan *et al.*, 1998) in which histological sections of normal or benign breast tissue were stained immunohistochemically for p53 (using the DO-7 antibody), we identified 29 subjects who showed p53 protein accumulation. One explanation for the p53 immunopositivity is that the tissue had an underlying p53 mutation. It is also possible that some of the 330 immunonegative subjects in that study had p53 mutations, since immunoreactivity can depend on the antibody used, on the type and duration of tissue fixation, or on the type of mutation, given that some mutations may not alter the protein in such a way that it can be detected immunohistochemically (Phillips *et al.*, 1999). In relation to the latter point, one study showed that approximately 33% of breast cancers with p53 gene mutations identified by complementary DNA sequencing did not show positive immunostaining in tissue sections using the Cl 1801 antibody (Sjögren *et al.*, 1996). In this study, we investigated whether the 29 p53 immunopositive breast tissue samples and 15 randomly selected p53 immunonegative breast tissue samples had p53 gene mutations.

MATERIAL AND METHODS

Clinical history and histopathology review

Breast tissue specimens from 44 women whose biopsies showed either no histopathological change or benign breast disease were analyzed. The women selected for the study had their biopsies performed between 1980 and 1987. For each patient a representative paraffin block containing tissue from the breast biopsy was obtained. Five-μm sections were cut, stained with hematoxylin and eosin, examined by light microscopy, and classified according to the criteria developed by Page and Anderson (1987).

p53 immunostaining

As described previously (Rohan *et al.*, 1998), 5-μm sections were cut from the paraffin blocks, mounted on aminopropyltri-

Grant sponsor: U.S. Army Medical Research and Material Command; Grant number: DAMD#17-96-1-6179.

*Correspondence to: Dr. Rita Kandel, Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, 600 University Avenue, Toronto, Ontario M5G 1X5 Canada. Fax: (416) 586-8628.
E-mail: rkandel@mtsinai.on.ca

Received 5 October 1999; Revised 17 January 2000

ethoxysilane (2%, Sigma, St. Louis, MO) coated slides and deparaffinized, and underwent antigen retrieval (microwaved in 10 mM citrate buffer, pH 6.0, for 15 min at a medium-high setting). Endogenous peroxidase was inactivated using 3% hydrogen peroxide, and the sections were blocked with goat serum (20 μl/ml, Vector, Burlingame, CA) containing 5% crystallized bovine serum albumin (BDH Laboratory Supplies, Poole, UK). The sections were incubated overnight at 4°C with antibody reactive with p53 (DO-7, dilution 1:40, Novocastra Laboratories, Newcastle Upon Tyne, UK). After washing, the sections were incubated with biotinylated goat anti-mouse IgG (dilution 1:200, Vector) for 30 min at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit, Vector). Immunoreactivity was visualized with 3',3-diaminobenzidine tetrahydrochloride (Vector) and the sections counterstained briefly with hematoxylin. The positive controls were sections from a paraffin-embedded breast cancer that was known to have a p53 mutation associated with p53 protein accumulation. The negative control consisted of replacing the primary antibody either with PBS or with mouse nonimmune serum. The presence of nuclear staining in any number of cells seen at 100× magnification was considered a positive reaction. Cytoplasmic staining was considered nonspecific and interpreted as negative.

p53 molecular analysis

Five-μm sections were cut from the paraffin blocks and stored for up to 3 years. Prior to microdissection, the sections were dewaxed and stained briefly with hematoxylin. The epithelium in the region of the tissue that had shown p53 immunoreactivity was microdissected out and placed in a microfuge tube. The tissue sections that showed no p53 protein accumulation immunohistochemically underwent random microdissection of epithelium. The tissue was digested with proteinase K (0.5 mg/ml in 50 mM Tris HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hr at 55°C. The proteinase K was inactivated by heating at 95°C for 15 min.

An aliquot of the digest was amplified using PCR, [α-^{33}P]-dATP and exon-specific primers (see Table I). An aliquot of the reaction product was separated on an 8% nondenaturing polyacrylamide gel, and the gel was processed for autoradiography. Potential mutations were detected by shifts in band mobility. If no band shifts were detected in these samples, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. If the two PCR-SSCP analyses generated different band shifts, another section was cut, microdissected, and processed for PCR-SSCP analysis as described above. Negative controls including cells that contained no mutation and a blank water control were included in each analysis. In addition, positive controls for exons 5 to 9 (exon 5: SKBr 3; exon 6: T47D; exon 7: colo 320 DM; exon 8: MDAMB468; exon 9: SW480) were also included where appropriate. The cell lines used as positive controls had been embedded in agar, fixed in 10% formalin, and were paraffin-embedded to simulate the processing conditions of the breast tissue.

The abnormally shifted band was excised from SSCP gels, and the DNA was eluted into water. The DNA was reamplified by PCR using the same primers, and the product was run on a 2% agarose gel. The band was extracted using QIAquick gel extraction kit (Qiagen, Mississauga, ON). The purified DNA was sequenced using ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, OH) and the sense primer according to the manufacturer's directions, followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Gene alterations were compared with those listed for breast cancer in a p53 database (http://www.iarc.fr/p53).

RESULTS

For two of the immunopositive cases, we were unable to extract DNA, and these cases were eliminated from the study. Of the 42 cases from which we could extract DNA, 22 showed fibrocystic change, 8 showed adenosis with or without fibrocystic change or fibrosis, 8 had hyperplasia (mild, moderate, or florid), 2 had fibroadenomas, and 2 showed no histopathological change.

Exons 4 to 10 were analyzed for mutations and a representative SSCP gel and its corresponding sequencing gel are shown in Figure 1. For all cases except one, the SSCP changes were reproducible. In the one case (case 24) where the SSCP change was not reproducible, the repeat analysis had been done on DNA extracted from a different section and only wild-type DNA sequences were seen on the second analysis. In all, 23 sequence alterations were detected in 20 individuals, and they were all base substitutions of the transition type (Tables II and III). Ten of these changes were missense mutations resulting in an amino acid change. Two of these mutations occurred at CpG dinucleotide sequences (cases 2 and 24), and 2 occurred at known hot spots on the p53 gene, one at codon 175 and the other at codon 245. The missense mutations were distributed amongst exons 4, 5, 7, and 9.

The other 13 gene changes (13/23) consisted of 8 silent (no amino acid change) base substitutions, 4 intronic base substitutions, and 1 uninterpretable change. The latter gene change occurred in case 5, for which an abnormal pattern for exon 9 was detected in the SSCP gel, while all the other exons showed wild-type patterns. Although the sequencing pattern could not be interpreted because of the presence of numerous extra bands, this sample was still considered to have a gene alteration. The 8 silent changes were detected in exons 4, 6, and 7, and the intronic changes were in introns 6, 7, and 9. Three individuals had 2 sequence changes each: in 2 of them both changes were silent and

TABLE I - P53 PRIMER SEQUENCES AND PCR CONDITIONS

Primers	Sequences	Product size (bp)	PCR conditions
Exon 4[1]	5'-ATCTACAGTCCCCCTTGCCG-3' 5'-GCAACTGACCGTGCAAGTCA-3'	296 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 35 cycles
Exon 5[2]	5'-GCTGCCGTGTTCCAGTTGCT-3' 5'-CCAGCCCTGTCGTCTCTCCA-3'	294 bp	95°C, 50 sec; 58°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 6[2]	5'-GGCCTCTGATTCCTCACTGA-3' 5'-GCCACTGACAACCACCCTTA-3'	199 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 7[2]	5'-TGCCACAGGTCTCCCCAAGG-3' 5'-AGTGTGCAGGGTGGCAAGTG-3'	196 bp	95°C, 50 sec; 56°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 8[2]	5'-CCTTACTGCCTCTTGCTTCT-3' 5'-ATAACTGCACCCTTGGTCTC-3'	225 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 9[3]	5'-GCCTCAGATTCACTTTTATCACC-3' 5'-CTTTCCACTTGATAAGAGGTCCC-3'	152 bp	95°C, 50 sec; 56°C, 50 sec; 72°C, 60 sec, 30 cycles
Exon 10[1]	5'-TGTTGCTGCAGATCCGTGGG-3' 5'-GAGGTCACTCACCTGGAGTG-3'	130 bp	95°C, 50 sec; 55°C, 50 sec; 72°C, 60 sec, 33 cycles

[1]Reference (Mashiyama *et al.*, 1991).–[2]Reference (Millikan *et al.*, 1995).–[3]Reference (Mazars *et al.*, 1992).

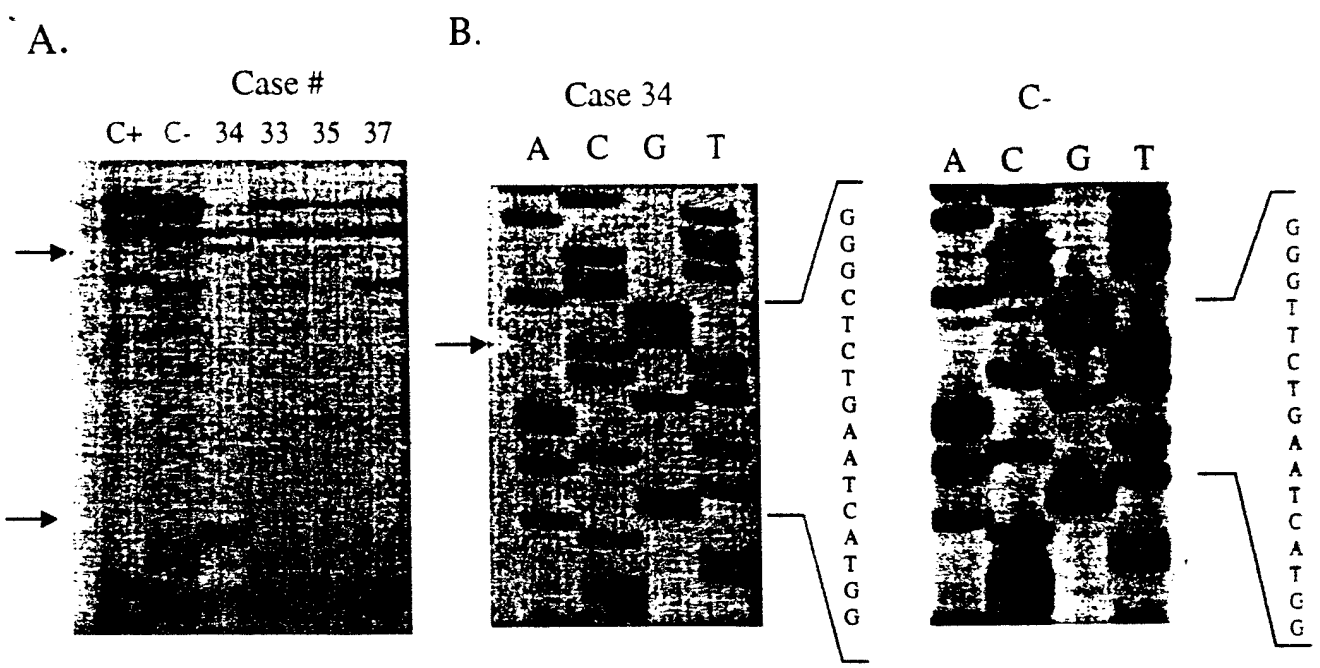

FIGURE 1 – (*a*) Representative SSCP gel of exon 9 PCR product from 4 cases. Case 34 shows a band shift as indicated by the arrow. The negative control that had wild-type p53 (C−) and positive (C+) control (cell line SW480) are included. (*b*) The corresponding sequencing gel of case 34 shows a base substitution (t→c) as indicated by the arrow. The sequencing pattern for the negative control (C−) in the same region is also shown.

TABLE II – P53 MUTATIONS IN BREAST TISSUE

Case number	p53 ICH[1]	Location	Codon	Sequence change	Amino acid
24	+	Exon 4	72	CGC → CGT	Arg → Arg
		Exon 4	110	CGT → TGT	Arg → Cys
46	+	Exon 4	76	GCA → ACA	Ala → Thr
2	+	Exon 5	175	CGC → CAC	Arg → His
4	+	Exon 5	135	TGC → TAC	Cys → Tyr
28	+	Exon 5	133	ATG → GTG	Met → Val
36	+	Exon 5	175	CAC → CGC	His → Arg
9	+	Exon 7	237	TCT → TTT	Ser → Phe
40	+	Exon 7	244	GGC → GAC	Gly → Asp
3	+	Exon 7	245	GGC → GAC	Gly → Asp
16	+	Exon 9	325	GGA → GAA	Gly → Glu

[1]ICH, immunohistochemical staining; +, present, −, absent.

in the third. 1 of the 2 resulted in an amino acid change. Of the 4 intronic alterations, 2 were in the same location (nucleotide residue 14766) in intron 5 and showed the same change (t→c). The others were at nucleotides 13466 in intron 6 and 14114 in intron 7. None of the intronic mutations occurred at a splice site or created a new splice site. In addition to the results shown in Tables II and III, the known p53 polymorphism in codon 72 (CGC→CCC) was detected in 2 cases.

All 10 missense mutations occurred in codons identified in the p53 breast cancer database as having mutations. Seven of them showed the same base and amino acid change as has been identified in breast cancer. Of the 8 silent changes, 3 showed the same base change as has been identified in breast cancer and 5 showed a different alteration in the same codon. A similar comparison could not be done for the intronic mutations because the nucleotide residues of the intronic mutations are not provided in the database. Similar to those reported in the p53 database, most base substitutions in this study were G:A and C:T (IARC p53 mutations database http://www.iarc.fr/p53).

For all individuals with a p53 gene alteration, the adjacent stromal tissue underwent microdissection and extraction of the DNA. The exon that had been identified as abnormal in the epithelial cells was analyzed by PCR-SSCP. In 18 of 20 samples/subjects, wild-type p53 banding patterns were observed (Fig. 2). In the other two (sample/subject 29 and 34), the same gene alteration was present in the stromal cells as in the epithelial cells.

Of the 27 breast tissue samples with p53 immunopositivity, 16 (59.2%) had p53 sequence changes. Nine of these 16 had mutations. One breast tissue with a p53 mutation was immunonegative (Table II). Seven of the 10 breast tissues with sequence alterations (silent or intronic) showed p53 immunoreactivity (Table III). Four of the 15 women (26.7%) whose biopsies were immunonegative showed sequence changes. One had a mutation (Table II) and 3 showed sequence alterations (Table III). There was no obvious association between the occurrence of gene alterations and any specific histopathologic features (Table IV). A representative photomicrograph of a section stained for p53 is shown in Figure 3.

DISCUSSION

p53 is involved in regulating cell proliferation and DNA repair, inducing apoptosis, and promoting chromosomal stability (Levine,

TABLE III - P53 SEQUENCE CHANGES THAT DO NOT CAUSE AMINO ACID CHANGES

Case number	p53 IHC[1]	Location	Site	Sequence	Amino acid
Silent change					
32	–	Exon 4	codon 74	GCC → GCT	Ala → Ala
		Exon 4	codon 111	CTG → CTA	Leu → Leu
44	+	Exon 4	codon 111	CTG → CTA	Leu → Leu
18	+	Exon 6	codon 217	GTG → GTA	Val → Val
27	+	Exon 7	codon 231	ACC → ACT	Thr → Thr
		Exon 7	codon 239	AAC → AAT	Asn → Asn
48	+	Exon 7	codon 226	GGC → GGT	Gly → Gly
Intronic change					
26	+	Intron 6	nr[2] 13466	g → a	
17	+	Intron 7	nr 14114	g → a	
29	–	Intron 9	nr 14766	t → c	
34	–	Intron 9	nr 14766	t → c	
Noninterpretable change					
5	+	Exon 9	–	–	

[1]IHC, immunohistochemical staining: +, present, –, absent.–[2]nr, nucleotide residue.

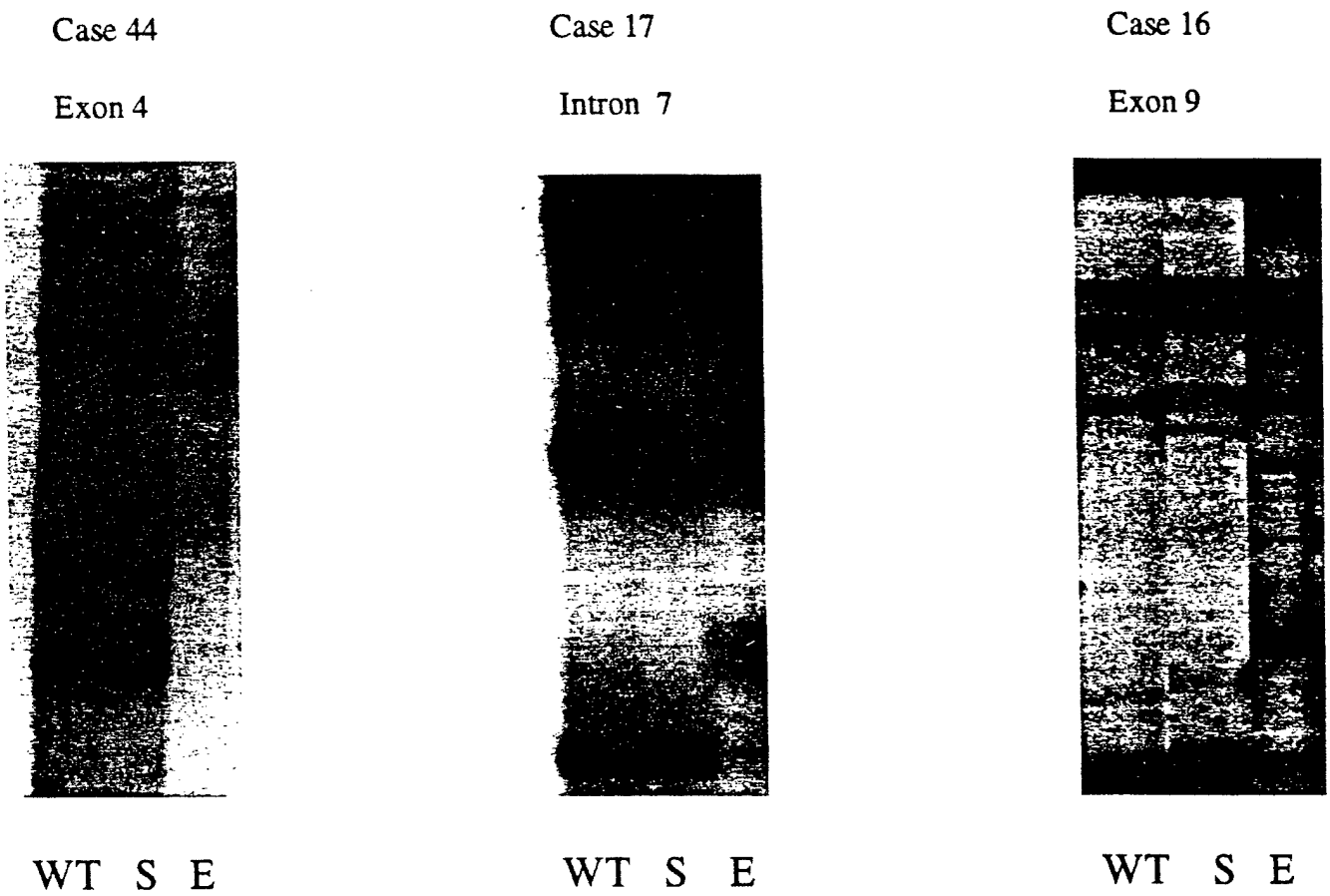

FIGURE 2 – Representative SSCP gels of exon 4 (case 44), intron 7 (case 17), and exon 9 (case 16) showing DNA that had been extracted from epithelial cells (E), from corresponding stromal cells (S), and the appropriate negative control (WT). The DNA from the epithelial cells shows a different band pattern than the corresponding stromal and negative control DNA.

1997). Changes in p53 might contribute to carcinogenesis by conferring a proliferative advantage to cells with or without abnormal DNA and/or by facilitating the accumulation of additional genetic changes, for example by allowing aneuploidy and genetic instability to occur (Shackney and Shankey, 1997). To date, it is not known at which stage in the carcinogenic process p53 abnormalities develop (Phillips *et al.*, 1999).

Our results demonstrate that p53 gene alterations can be detected in breast tissue that is either normal or shows changes of benign breast disease. p53 changes were found more commonly in tissue that showed p53 protein accumulation (positive immunostaining) than in tissue that did not. All of the changes detected were of the transition type. This is in keeping with experimental data showing that DNA proofreading corrects transversions more efficiently than transitions (Schaaper, 1993).

There have been 3 other reports of p53 gene analysis in normal or benign breast tissue. Millikan *et al.* (1995) detected p53 point mutations in 5 of 90 paraffin-embedded breast samples. Two of the

TABLE IV - SUMMARY OF ANALYSES OF P53 IMMUNOHISTOCHEMICALLY DETECTED PROTEIN ACCUMULATION AND GENE CHANGES ACCORDING TO HISTOLOGICAL FEATURES

Histology	Number of samples	Number of samples I+ M+	Number of samples I+ M−	Number of samples I− M+	Number of samples I− M−
Normal	2	2	0	0	0
FCC[3]	22	9	5	2	6
Adenosis[4]	8	1	4	1	2
Hyperplasia[5]	8	3	2	1	2
Fibroadenoma	2	1	0	0	1
Total	42	16	11	4	11

[1]I+, immunopositive; I−, immunonegative.–[2]M+, gene change present; M−, gene change absent.–[3]FCC, fibrocystic change.–[4]Adenosis, adenosis ± FCC ± fibrosis.–[5]Hyperplasia, either mild, moderate, or florid.

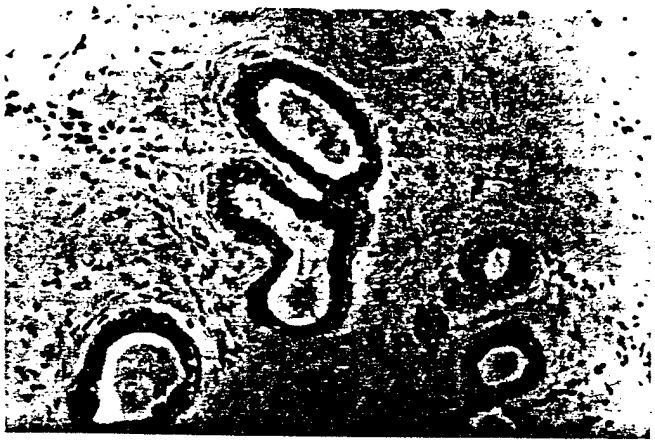

FIGURE 3 – Photomicrograph of normal breast ducts showing p53 immunopositivity (immunoperoxidase with hematoxylin counterstain, magnification 160×).

mutations occurred within the 14 cases that were immunopositive for p53 and the other 3 occurred in the 46 immunonegative cases. All of the mutations were transition types, and 3 resulted in an amino acid change. In contrast to our study, their analysis involved only exons 4 to 8, which may provide a partial explanation for the lower frequency of mutations in their study. Lisboa *et al.* (1997) detected a p53 mutation in one of 13 cases of normal or benign breast tissue examined. However, they examined only exons 5 to 8 inclusive and did not perform microdissection and so may have missed mutation(s) present in only a small number of cells. Done *et al.* (1998) identified 7 cases of breast cancer from which they were able to microdissect 41 foci of surrounding normal epithelium or epithelium showing changes of benign breast disease. The p53 gene analysis was performed on DNA extracted from paraffin-embedded tissue. They did not detect any mutations, but their study was based on a small number of cases and only exons 4 to 8 were studied.

In this study, sequence changes occurred overall in 59.2% (16/27) of p53 immunopositive samples. p53 mutations occurred in 33% (9/27) of immunopositive cases. Although this value may appear low, it is in keeping with the findings of several studies of breast cancer, which have examined the correlation between immunostaining and the presence of mutations detected by sequencing. In those studies, 20% (Dunn *et al.*, 1993) to 70% (Visscher *et al.*, 1996) of immunopositive breast cancers showed mutations. The relatively low value that we observed may in part reflect the fact that we considered the presence of any p53 immunopositivity to represent a positive case, whereas it has been suggested by others that only cases showing immunopositivity in greater than 5% of cells should be considered to have p53 protein accumulation (Clausen, 1998). It is also possible that more mutations may have been identified if the entire coding region (exons 2 to 11) and not just exons 4 to 10 had been sequenced. Alternatively, the p53 protein accumulation may be due to mechanisms other than p53 mutation. Four (26.7%) of our p53 immunonegative cases showed gene alterations, one of which was a mutation. It is not surprising that p53 changes were detected in the absence of positive immunostaining as it is well accepted that not all p53 mutations will result in immunohistochemically detectable p53 protein (Sjögren *et al.*, 1996; Visscher *et al.*, 1996).

Several features of our study suggest that the mutations that were detected were real and were not artifacts of the methodology used to detect them. It has been shown that PCR-induced sequence changes can be minimized if the proteinase digestion time of the tissue is sufficiently prolonged (at least 48 hr), the products generated by PCR are relatively small (Shiao *et al.*, 1997), and enough DNA template is used (Krawczak *et al.*, 1989). In our study, the tissue was digested for at least 48 hr and the products were all less than 300 bp in size. Although we were unable to quantify the amount of DNA in each analysis, a fixed cycle number was used in the PCR for each exon of all samples and it was not increased if the product was undetectable. Secondly, repeat PCR-SSCP analysis showed that the band shifts were reproducible. In the one case where it was not reproducible, the analysis had been done on DNA extracted from a different section and it is likely that the area with the p53 change was no longer present. The DNA in the abnormal SSCP band was sequenced in both directions to ensure that the sequence change was not a PCR-induced artifact and in each case the same mutation(s) was detected. Thirdly, DNA from stromal tissue showed wild-type p53 sequences in 18 of 20 cases. The other 2 cases (29 and 34) had the same mutation in both epithelial and stromal DNA. For these samples, the changes might represent inadvertent microdissection of some epithelial cells with the stromal tissue or a true germline mutation or a polymorphism. We consider it more likely that the change detected in these two is a polymorphism because it has been detected in approximately 4% of tumours in a breast cancer tumour bank (data not shown). Fourthly, we were able to detect a known polymorphism in 2 other cases. Finally, other studies, such as that of Nadji *et al.* (1996), have shown that DNA extracted from paraffin-embedded tissue will show p53 gene changes identical to those detected in frozen tissue, suggesting that paraffin-embedding does not induce gene mutations and that tissue processed in this way is suitable for DNA analysis. Eight (34.8%) of the 23 gene changes detected were silent. Strauss (1997) predicted that approximately 25% of mutations in a dataset will be silent if mutagenesis is random and if the silent mutation does not provide a selective advantage. Although the significance of silent mutations is not known, it is possible that they could have effects on DNA.

In conclusion, the results of this study suggest that p53 mutations can be detected in normal epithelium and benign breast tissue. This observation is in keeping with the findings of other studies demonstrating genetic changes such as loss of heterozygosity (LOH) and microsatellite instability in normal and benign breast tissue (Deng *et al.*, 1996; Lakhani *et al.*, 1996; O'Connell *et al.*, 1998; Larson *et al.*, 1998). However, the significance of p53

mutations and other sequence changes in these tissue types is unknown. For example, a study showed that genetic changes such as LOH and microsatellite instability may not correlate with the development of breast cancer (Kasami *et al.*, 1997). For skin, however, it has been suggested that p53 mutations may provide information about subsequent risk of developing nonmelanoma skin cancer (Ouhtit *et al.*, 1998). Resolution of the role of p53 gene alterations in the pathogenesis of breast cancer may require long-term follow-up studies involving comparisons of breast cancer occurrence in patients with and without p53 mutations and assessment of the functional significance of the mutations.

ACKNOWLEDGEMENTS

We thank Ms. L. Cutler for her excellent secretarial assistance.

REFERENCES

BARNES, D.M. and 13 OTHERS, Abnormal expression of wild type p53 protein in normal cells of a cancer family patient. *Lancet*, **340**, 259–263 (1992).

CAMPOMENOSI, P., CONIO, M., BOGLIOLO, M., URBINI, S., ASSERETO, P., APELE, A., MONTI, P., ASTE, H., LAPERTOSA, G., INGA, A., ABBONDANDOLO, A. and FRONZA, G., p53 is frequently mutated in Barrett's metaplasia of the intestinal type. *Cancer Epidemiol. Biomarkers Prev.*, **5**, 559–565 (1996).

CLAUSEN, O.P., LOTHE, R.A., BORRESEN-DALE, A-L., DE ANGELIS, P., CHEN, Y., ROGNUM, T.O. and MELING, G.I., Association of p53 accumulation with TP53 mutations, loss of heterozygosity at 17p13, and DNA ploidy status in 273 colorectal carcinomas. *Diagn. Mol. Pathol.*, **7**, 215–223 (1998).

DENG, G., LU, Y., ZLOTNIKOV, G., THOR, A.D. and SMITH, H.S., Loss of heterozygosity in normal tissue adjacent to breast carcinomas. *Science*, **274**, 2057–2059 (1996).

DONE, S.J., ARNESON, N.C.R., OZCELIK, H., REDSTON, M. and ANDRULIS, I.L., p53 mutations in mammary ductal carcinoma in situ but not in epithelial hyperplasias. *Cancer Res.*, **58**, 785–789 (1998).

DUNN, J.M., HASTRICH, D.J., NEWCOMB, P., WEBB, J.C.J., MAITLAND, N.J. and FARNDON, J.R., Correlation between p53 mutations and antibody staining in breast carcinoma. *Brit. J. Surg.*, **80**, 1410–1412 (1993).

GAO, Y., GAO, Q., WAZER, D.E. and BAND, V., Abrogation of wild-type p53-mediated transactivation is insufficient for mutant p53-induced immortalization of normal human mammary epithelial cells. *Cancer Res.*, **57**, 5584–5589 (1997).

GAO, Q., HAUSER, H., LIU, X-L., WAZER, D.E., MADOC-JONES, H. and BAND V., Mutant p53-induced immortalization of primary human mammary epithelial cells. *Cancer Res.*, **56**, 3129–3133 (1996).

JERRY, D.J., OZBUN, M.A., KITTRELL, F.S., LANE, D.P., MEDINA, D. and BUTEL, J.S., Mutations in p53 are frequent in the preneoplastic stage of mouse mammary tumor development. *Cancer Res.*, **53**, 3374–3381 (1993).

KASAMI, M., VNENCAK-JONES, C.L., MANNING, S., DUPONT, W.D. and PAGE, D.L., Loss of heterozygosity and microsatellite instability in breast hyperplasia. No obligate correlation of these genetic alterations with subsequent malignancy. *Amer. J. Pathol.*, **150**, 1925–1932 (1997).

KRAWCZAK, M., REISS, J., SCHMIDTKE, J. and ROSLER, U., Polymerase chain reaction: replication errors and reliability of gene diagnosis. *Nucl. Acid Res.*, **17**, 2197–2201 (1989).

LAKHANI, S.R., SLACK, D.N., HAMOUDI, R.A., COLLINS, N., STRATTON, M.R. and SLOANE, J.P., Detection of allelic imbalance indicates that a proportion of mammary hyperplasia of usual type are clonal, neoplastic proliferations. *Lab. Invest.*, **74**, 129–135 (1996).

LARSON, P.S., DE LAS MORENAS, A., CUPPLES, L.A., HUANG, K. and ROSENBERG, C.L., Genetically abnormal clones in histologically normal breast tissue. *Am. J. Pathol.*, **152**, 1591–1598 (1998).

LEVINE, A., p53, the cellular gatekeeper for growth and division. *Cell*, **88**, 323–331 (1997).

LI, B., MURPHY, K.L., LAUCIRICA, R., KITTRELL, F., MEDINA, D. and ROSEN, J.M., A transgenic mouse model for mammary carcinogenesis. *Oncogene*, **16**, 997–1007 (1998).

LISBOA, B.W., VOGTLANDER, S., GILSTER, T., RIETHDORF, L., MILD-LANGOSCH, K. and LONING, T., Molecular and immunohistochemical analysis of p53 mutations in scrapings and tissue from preinvasive and invasive breast cancer. *Virchows Arch.*, **431**, 375–381 (1997).

MASHIYAMA, S., MURAKAMI, Y., YOSHIMOTO, T., SEIKIYA T. and HAYASHI, K., Detection of p53 gene mutations in human brain tumors by single-strand conformation polymorphism analysis of polymerase chain reaction products. *Oncogene*, **6**, 1313–1318 (1991).

MAZARS, R., SPINARDI, L., BENCHEIKH, M., SIMONY-LAFONTAINE, J., JEANTEAUR, P. and THEILLET, C., p53 mutations occur in aggressive breast cancer. *Cancer Res.*, **52**, 3918–3923 (1992).

MILLIKAN, R. and 12 OTHERS, p53 mutations in benign breast tissue. *J. Clin. Oncol.*, **13**, 2293–2300 (1995).

NADJI, M., MENG, L., LIN, L., NASSIRI, M. and MORALES, A.R., Detection of p53 gene abnormality by sequence analysis of archival paraffin tissue. *Diagn. Mol. Pathol.*, **5**, 279–283 (1996).

O'CONNELL, P., PEKKEL, V., FUQUA, S.A.W., OSBORNE, C.K., CLARK, G.M. and ALLRED, D.C., Analysis of loss of heterozygosity in 399 premalignant breast lesions at 15 genetic loci. *J. Natl. Cancer Inst.*, **90**, 697–703 (1998).

OUHTIT, A., NAKAZAWA, H., ARMSTRONG, B.K., KRICKER, A., TAN, E., YAMASAKI, H. and ENGLISH, D.R., UV-radiation-specific p53 mutation frequency in normal skin as a predictor of risk of basal cell carcinoma. *J. Natl. Cancer Inst.*, **90**, 523–531 (1998).

PAGE, D.L. and ANDERSON, T.J., *Diagnostic histopathology of the breast*, Churchill Livingstone, New York (1987).

PHILLIPS, H.A., The role of the p53 tumour suppressor gene in human breast cancer. *Clin. Oncol.*, **11**, 148–155 (1999).

ROHAN, T.E., HARTWICK, W., MILLER, A.B. and KANDEL, R., Immunohistochemical-detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. *J. nat. Cancer Inst.*, **90**, 1262–1269 (1998).

SCHAAPER, M., Base selection, proofreading, and mismatch repair during DNA replication in *Eschericia coli*. *J. Biol. Chem.*, **268**, 23762-23765 (1993).

SCHMITT, F.C., LEAL, C. and LOPES, C., p53 protein expression and nuclear DNA content in breast intraductal proliferations. *J. Pathol.*, **176**, 233–241 (1995).

SHACKNEY, S.E. and SHANKEY, T.V., Common patterns of genetic evolution in human solid tumors. *Cytometry*, **29**, 1–27 (1997).

SHIAO, Y-H., BUZARD, G.S., WEGHORST, C.M. and RICE, J.M., DNA template as a source of artifact in the detection of p53 gene mutations using archived tissue. *Biotechniques*, **4**, 608–612 (1997).

SJÖGREN, S., INGANÄS, M., NORBERG, T., LINDGREN, A., NORDGREN, H., HOLMBERG, L. and BERGH, J., The p53 gene in breast cancer: prognostic value of complementary DNA sequencing versus immunohistochemistry. *J. Natl. Cancer Inst.*, **88**, 173–182 (1996).

STRAUSS, B.S., Silent and multiple mutations in p53 and the question of the hypermutability of tumors. *Carcinogenesis*, **18**, 1445–1452 (1997).

THOR, A.D., MOORE, D.H., EDGERTON, S.M., KAWASAKI, E.S., REIHSAUS, E., LYNCH, H.T., MARCUS, J.N., SCHWARTZ, L., CHEN, L-C., MAYALL, B.H. and SMITH, H.S., Accumulation of p53 tumor suppressor gene protein: an independent marker of prognosis in breast cancers. *J. Natl. Cancer Inst.*, **84**, 845–855 (1992).

YOUNES, M., LEBOVITZ, R.M., BOMMER, K.E., CAGLE, P.T., MORTON, D., KHAN, S. and LAUCIRICA, R., p53 accumulation in benign breast biopsy specimens. *Hum. Pathol.*, **26**, 155–158 (1995).

VISSCHER, D.W., SARKAR, F.H., SHIMOYAMA, R.K. and CRISSMAN, J.D., Correlation between p53 immunostaining patterns and gene sequence mutations in breast carcinoma. *Diagn. Mol. Pathol.*, **5**, 187–193 (1996).

Cyclin D1 Protein Overexpression and Gene Amplification in Benign Breast Tissue and Breast Cancer Risk

Rita Kandel[1], M.D., Xin Li Zhu[1], PhD., Shu-Qui Li[1], and Tom Rohan[2], M.D., PhD.

[1]Department of Pathology and Laboratory Medicine, Mount Sinai Hospital
University of Toronto, Toronto, Canada

[2]Department of Epidemiology and Social Medicine, Albert Einstein College of Medicine, Bronx, New York (TR)

Send correspondence to:
Dr. Rita Kandel
Department of Pathology and Laboratory Medicine
Mount Sinai Hospital, Room 600
600 University Avenue
Toronto, Ontario M5G 1X5
Tele: 416-586-8516
Fax: 416-586-8628
email: rkandel@mtsinai.on.ca

Running Title: Cyclin D1 and breast cancer risk

ACKNOWLEDGEMENTS

We thank Lori Cutler and Isabelle Schell for their secretarial assistance. This work was supported by the US Army Medical Research and Materiel Command and was presented in part at the American Association for Cancer Research, Philadelphia, PA, April, 1-5, 2000.

ABSTRACT

Cyclin D1 amplification and/or protein overexpression have been observed not only in breast cancer but also in the putative early stages of breast neoplasia. In a case-control study nested within a cohort of 4,888 women, we investigated whether the occurrence of cyclin D1 gene amplification and/or protein overexpression in benign breast tissue might identify women at increased risk of subsequent breast cancer development. Cases were 92 women with a histological diagnosis of benign breast disease who subsequently developed breast cancer. Five controls (women with benign breast disease who had not developed breast cancer by the date of diagnosis of the corresponding case) were selected randomly for each case from those non-cases available within strata defined by screening center, NBSS study arm, year of birth, and age at diagnosis of benign breast disease. Paraffin blocks of benign tissue were suitable for immunostaining for 71 cases and 293 controls. Sufficient DNA for analysis was obtained from a total of 356 subjects (69 cases, 287 controls). The benign breast tissues and breast cancers were immunostained for cyclin D1and also analyzed for the presence of cyclin D1 gene amplification by differential PCR. 15 cases and 60 controls showed evidence of cyclin D1 immunostaining, and 12 cases and 29 controls showed cyclin D1 gene amplification. There was essentially no association between cyclin D1 protein overexpression in benign breast tissue and risk of subsequent breast cancer (adjusted OR = 1.06, 95% CI = 0.56-2.02). After adjustment for potential confounding, there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 gene amplification (adjusted OR = 1.41, 95% CI = 0.62-3.22). As multiple genetic changes are required to develop breast cancer, it may not be until the cascade of molecular alterations leading to breast cancer development are known that identification of biomarkers of breast cancer risk will be identified.

Key Words: cyclin D1, gene amplification, protein overexpression, benign breast tissue

INTRODUCTION

In non-tumourous cells, cyclin D1, together with the cyclins D2 and D3, is involved in regulating progression from G_1 to the S phase of the cell cycle (Barnes and Gillett, 1998, Steeg and Zhou, 1998) These cyclins form complexes with cdk4 or cdk6 which can then phosphorylate the retinoblastoma (Rb) protein, resulting in the release of E2F transcription factors and allowing cells to progress into the S phase. Cyclin D1 has other regulatory effects. Specifically, it has been implicated in the replication and repair of DNA, as it can bind to PCNA [proliferating cell nuclear antigen] (Xiong et al., 1992), a protein involved in DNA synthesis, and cells that overexpress cyclin D1 are unable to repair DNA damage induced by ultraviolet radiation (Pagano et al., 1994). Experimentally, a transfected rat liver cell line that overexpresses cyclin D1 showed an increased number of cells with CAD amplification, suggesting that under specific conditions cyclin D1 may enhance gene amplification and contribute to genomic instability (Zhou et al., 1996)

Given the multifarious effects of cyclin D1 on cell function and genomic integrity, it might be postulated that perturbations of normal cyclin D1 levels are related to altered cancer risk. (Zhou et al., 2000). Indeed, since cyclin D1 amplification and/or protein overexpression have been observed not only in breast cancer (Buckley et al., 1993, Zukerberg et al., 1995, Zhang et al., 1994, Frierson et al., 1996, Barbareschi et al., 1997) but also in the putative early stages of breast neoplasia (Weinstat-Saslow et al., 1995, Simpson et al., 1997, Alle et al., 1998, Zhu et al., 1998, Gillett et al., 1998), it might be postulated that such changes contribute to breast cancer development. Therefore, in the cohort study reported here, we investigated whether the occurrence of cyclin D1 gene amplification and/or protein overexpression in benign breast tissue might identify women at increased risk of subsequent breast cancer development.

MATERIALS AND METHODS

Subjects and Methods

The study methods have been described in detail elsewhere (Rohan et al., 1998). In brief, the investigation was undertaken as a case-control study nested within the cohort of 4,888 women in the National Breast Screening Study (NBSS) who received a histopathologic diagnosis of benign breast disease during the active follow-up phase of the NBSS. The NBSS is a multi-center randomized controlled trial of screening for breast cancer in 89,835 Canadian women who were recruited between 1980 and 1985, and who were followed actively until 1988 and passively thereafter (Miller et al., 1981, Miller et al., 1992). Women were eligible to participate if they were 40-59 years old and had no previous history of breast cancer (in situ or invasive).

Diagnosis of Breast Disease in the NBSS

In the NBSS, patients with clinical or radiological evidence of a lesion underwent either needle aspirates or biopsies. For those subjects who had biopsies, the histological sections were reviewed for study purposes by a reference pathologist. The study reported here was restricted to subjects who had no evidence of breast cancer (in situ or invasive) in their initial surgical biopsy as determined on review by an NBSS reference pathologist. Women with a history of previous benign breast disease were not excluded from participation. The characteristics of the cohort have been described previously (Rohan at al., 1998).

Ascertainment of Outcome

Incident cases of breast cancer were ascertained by record linkage with the provincial cancer registries, and a death clearance was performed by linkage to the Canadian National Mortality Database (Miller et al., 1992). The dates of the linkages varied by province, ranging from the end of 1988 to early 1991.

Definition of Cases

Cases were the 92 women with a histological diagnosis of benign breast disease made by a reference pathologist during the active follow-up phase of the NBSS and who subsequently developed breast cancer. In this study, cancer was defined as any form of breast carcinoma: there were 16 cases with ductal carcinoma in situ (DCIS) and 76 cases with invasive carcinoma.

Definition and Selection of Controls

Controls were women with benign breast disease who had not developed breast cancer by (but were alive at) the date of diagnosis of the corresponding case. Five controls were selected randomly for each case from those non-cases available within strata defined by screening center, NBSS study arm, year of birth, and age at diagnosis of benign breast disease.

Questionnaires

At the time of their enrolment in the NBSS, all participants completed a questionnaire which sought data on potential breast cancer risk factors, including demographic characteristics, family history of breast cancer, and menstrual and reproductive history.

Acquisition of Paraffin-embedded Blocks of Breast Tissue

For the present study, hospitals and clinics storing the paraffin-embedded blocks of benign and malignant tissue were asked to send one representative block per lesion and to indicate the fixative type and whether the tissue had been frozen prior to fixation. Blocks or sections of paraffin-embedded benign tissue were obtained for 74 (80.4%) of the 92 cases and for 349 (75.8%) of the 460 controls; blocks or sections of malignant tissue were obtained for 62 (83.8%) of the 74 cases (Rohan et al., 1998).

Histopathology Review

Sections from the blocks received were reviewed and classified according to the criteria developed by Page and Anderson (Page and Anderson, 1987) and the recent consensus

conference (Schwartz et al, 1997), without knowledge of the case-control status of the study subjects.

Breast Cancer Cell Lines

The human breast carcinoma derived cell lines ZR-75-1, which has two to five-fold amplification of cyclin D1 (Bartkova et al., 1994), MDA-MB-231, which shows no cyclin D1 gene amplification (Frierson et al, 1996), and T47D, which shows cyclin D1 overexpression immunohistochemically (Bartkova et al., 1994), were obtained from the American Type Culture Collection (ATCC). The cells were grown in culture, harvested using trypsin - EDTA (Sigma Chemical Co., St. Louis, MO), and centrifuged to form pellets. The cell pellets were placed in 3% bacto-agar (Difco Laboratories, Detroit, MI), fixed in 10% buffered formalin and then embedded in paraffin. Five μm thick sections were cut and used as controls for the polymerase chain reaction (PCR) and/or immunostaining.

Cyclin D1 Immunostaining

Immunostaining was performed as described previously (Zhu et al., 1998). Briefly, tissue sections which had been stored for up to three years underwent antigen retrieval (microwave pretreatment in 10 mM citrate buffer, pH 6.0, for 15 minutes at a medium-high setting) and were incubated overnight at 4°C with antibody reactive with cyclin D1 protein (monoclonal, dilution 1:2000; Upstate Biotechnology, Lake Placid, NY). After washing, the sections were incubated with biotinylated antimouse immunoglobulin G (dilution 1:200; Vector Laboratories) for 30 minutes at room temperature, followed by avidin-biotin peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories). Immunoreactivity was visualized with 3,3'-diaminobenzidine (Vector), and the sections were counterstained briefly with hematoxylin. T47D cells embedded in paraffin served as the positive control. The negative control consisted of replacing the primary antibody with Tris-buffered saline or nonimmune mouse serum (DAKO, Carpinteria,

CA). Distinct nuclear staining in greater than 1% of epithelial cells indicated a positive reaction and cytoplasmic staining was considered nonspecific and interpreted as negative.

Determination of Cyclin D1 Gene Amplification

Tissue microdissection: Five μm sections were stained briefly with hematoxylin to visualize the epithelium. The sections were matched to the corresponding immunostained sections. The epithelium in the area of the tissue corresponding to that which had shown cyclin D1 immunoreactivity was microdissected out and placed in a microfuge tube. If the tissue had shown no immunostaining for cyclin D1 the corresponding section underwent random microdissection of epithelium. DNA was extracted by incubating the microdissected tissue in buffer (50 mM Tris-HCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) containing 0.5 mg/ml of proteinase K (Sigma Chemical Co, St. Louis, MO) at 55°C for 48 hours. The proteinase K was then inactivated by heating at 95°C for 15 minutes.

Differential polymerase chain reaction: Semiquantitative differential polymerase chain reaction (PCR) was used to determine the presence of cyclin D1 gene amplification (Zhu et al., 1998). As fragmented genomic DNA (<200 bp) may influence the results of differential PCR, γ-interferon (γ-IFN), which is a single copy gene, was analyzed in a multiplex PCR reaction in order to indirectly assess DNA quality first, as described previously (Zhu et al., 1998, Frye et al., 1989, Neubauer et al., 1992). If the γ-IFN82/γ-IFN150 ratio of the PCR products was three or less, the tissue was considered suitable for further analysis. For such cases, aliquots of the proteinase K digested tissue were then examined for cyclin D1 amplification using PCR. If chromosomal aneuploidy is present within the tissue it might simulate amplification, resulting in a false positive result. To prevent this we selected the dopamine receptor (DR) for co-amplification, as it is present on the same chromosome as cyclin D1 (Grandy et al., 1989, Gramlich et al., 1994), yet of sufficient distance from cyclin D1 that it is unlikely to be part of an amplified amplicon. Each

run included DNA extracted from paraffin-embedded cell lines, MDA-MB-231 (negative control) and ZR-75-1 (positive control).

Briefly, 1 µl of the digest was mixed with 14 µl of PCR working solution containing 50 mM Tris HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 100 µM of each dNTP, 1 U of AmpliTaq DNA polymerase (Roche Diagnostic Systems Inc., Branchburg, NJ) and 0.4 µM of each primer. The primers and PCR conditions are shown in Table 1. The PCR products were separated on a 12% polyacrylamide gel at 200V for two hours and visualized following ethidium bromide staining. DNA from each sample was analyzed at least twice in separate polymerase chain reactions. Samples showing reproducible amplification of cyclin D1, then underwent a third PCR which included samples of DNA that had been extracted from the subject's breast stromal tissue. As cyclin D1 is not amplified in this tissue, it served as an internal control for the presence of cyclin D1 amplification in the breast epithelium. Direct sequencing of selected PCR products using the sense primer and the ThermoSequenase radiolabelled terminator cycle sequencing kit (Amersham Life Sciences, Cleveland, Ohio, USA) confirmed that the product was cyclin D1 as described previously (Zhu et al., 1998).

Quantification of cyclin D1 amplification: To determine whether there was cyclin D1 gene amplification, the ratio of the cyclin D1 PCR product to the *DR* PCR product was derived from photographic negatives of ethidium bromide stained gels. The bands were quantified by laser densitometry (Computing Densitometer Model 300A, Molecular Dynamics, Sunnyvale, CA). There were at least two gels per PCR product and each gel was scanned two times. A mean ratio of cyclin D1 to DR of greater than 0.88 was considered indicative of gene amplification. This value was determined by identifying the point two standard deviations above the average of the ratios (n=93) obtained from the control cell line, MDA-MB-231, that had no gene amplification.

c-erbB-2 Protein Overexpression and p53 Protein Accumulation

Immunostaining for c-erbB-2 and p53 was performed as described previously (Rohan et al., 1998).

Statistical Analysis

Odds ratios (OR) and 95 percent confidence intervals (CI) for the associations between cyclin D1 protein overexpression and gene amplification and risk of breast cancer were obtained from conditional logistic regression models (Breslow and Day, 1980). Adjusted odds ratio estimates were obtained by including terms representing the following potential confounders in the regression models: history of breast cancer in a first degree relative, age at menarche, age at first live birth, menopausal status (pre-, peri-, and post-menopausal), body mass index (weight(kg)/height(m)^2), and hyperplasia (ductal or lobular, with or without atypia). For categorical variables, tests for trend (on one degree of freedom) in associations were performed by fitting the categorized variables as continuous variables in conditional logistic regression models. Further analyses included within individual comparisons of cyclin D1 in benign breast disease and breast cancer. All statistical tests were two-sided.

RESULTS

As described previously (Rohan et al., 1998), blocks of benign tissue were obtained for 74 (80.4%) of the 92 cases and for 349 (75.9%) of the 460 controls; however, blocks were stained for 309 of the controls only, since for 40 controls benign tissue was not obtained for the corresponding case. For three cases and 15 controls, the benign tissue was inadequate for immunohistochemical analyses, and therefore the statistical analyses were based on 71 cases and 293 controls. DNA was extracted from paraffin blocks and yielded sufficient DNA suitable for analysis in a total of 356 subjects (69 cases, 287 controls).

As shown previously (Rohan et al., 1998) in this study population, risk of breast cancer was altered little in association with a family history of breast cancer, age at menarche, age at first livebirth, menopausal status, Quetelet's index, and the presence of hyperplasia in benign tissue. However, the patterns of risk were mostly in accord with expectation. Also, there were few differences between those subjects for whom benign tissue was and was not obtained with respect to their distributions by breast cancer risk factors.

Of the subjects whose benign tissue was suitable for immunohistochemical analysis, 15 cases and 60 controls showed evidence of cyclin D1 immunostaining (Figure 1 and Table 2). There was essentially no association between cyclin D1 protein overexpression in benign breast tissue and risk of subsequent breast cancer, and there was little variation in risk by the percentage of cells showing immunostaining. Twelve cases and 29 controls showed cyclin D1 gene amplification (Figure 2 and Table 2). After adjustment for potential confounding, there was a statistically non-significant 40% increase in risk of breast cancer in association with cyclin D1 gene amplification. For those with hyperplasia and cyclin D1 immunostaining, the adjusted OR was 1.66 (95% CI = 0.75-3.71); for those with hyperplasia and cyclin D1 gene amplification, the adjusted OR was 1.55 (95% CI = 0.52-4.69). Compared to those with neither cyclin D1

immunostaining nor cyclin D1 gene amplification, the adjusted OR (95% CI) for those with either or both of these changes were 1.47 (0.71-3.03) and 0.99 (0.34-2.90), respectively. As estrogens regulate cyclin D1 expression (Prall OW et al., 1998) cyclin D1 levels might vary during the menstrual cycle. However, additional adjustment for days since last menstrual period had little effect on the odds ratio reported in table 2 (data not shown).

After exclusion of the 19 cases (and their matched controls) whose diagnosis of breast cancer occurred within one year of their diagnosis of benign breast disease, the adjusted OR (95% CI) for the associations between cyclin D1 protein overexpression and gene amplification and risk of breast cancer were 1.15 (0.53-2.50) and 2.27 (0.90-5.71), respectively. When the analyses were restricted to the matched case-control sets containing cases with invasive breast cancer (that is, after exclusion of the 14 cases with DCIS and their matched controls), the adjusted OR for cyclin D1 immunopositivity was 1.29 (95% CI = 0.58-2.87), while the adjusted OR for cyclin D1 gene amplification was 1.79 (95% CI = 0.66-4.86). Also, when the 23 cases whose benign and malignant lesions occurred in opposite breasts were excluded, the adjusted ORs for cyclin D1 immunopositivity and gene amplification were 1.08 (95% CI = 0.47-2.48) and 1.55 95% CI = 0.53-4.52), respectively. As well, the results for cyclin D1 immunostaining and gene amplification did not differ between strata defined by age, menopausal status, NBSS study arm, history of previous breast disease, and whether the benign breast disease was screen-detected or interval detected.

As described elsewhere (Rohan et al., 1998), risk of breast cancer was increased in those with positive immunostaining for p53 in their benign breast tissue but not in those with immunostaining for c-erbB-2. When risk was examined according to the number of markers for which positive immunostaining was observed (relative to the risk in those with negative immunostaining for all three markers), the adjusted OR (95% CI) associated with positive

immunostaining for one only and for two or more markers (only one case and one control had positive immunostaining for all three markers) were 1.19 (0.63-2.23) and 0.96 (0.33-2.81), respectively.

Table 3 shows the concordance between the immunohistochemical and gene amplification findings for the benign and malignant tissue for the cases. Of the 39 subjects who were negative for cyclin D1 protein overexpression in their benign tissue, about 31% (12/39) showed evidence of overexpression in their malignant tissue; four (28.6%) of the 14 subjects with immunostaining in their benign tissue had cancers which did not show immunostaining. For gene amplification, the corresponding values were 21.2% (7/33) and 77.8% (7/9).

DISCUSSION

Cyclin D1 gene amplification and/or protein expression has been detected in non-cancerous breast tissue. Breast epithelium that is either normal or has changes of benign breast disease, including breast papillomas, can show cyclin D1 protein overexpression immunohistochemically (Alle et al., 1998, Zhu et al., 1998, Gillett et al., 1998, Saddik et al., 1999). The frequency of cyclin D1 protein overexpression is greater in proliferative disease with atypia than in normal epithelium or in the presence of proliferative disease without atypia, as demonstrated in two studies (Alle et al., 1998, Zhu et al., 1998). In one of those studies (Zhu et al., 1998), cyclin D1 gene amplification was also examined and was detected in normal and benign breast tissue. In an in-situ hybridization study, 18% of benign breast lesions showed cyclin D1 mRNA overexpression (Weinstat-Saslow et al., 1995). Cyclin D1 gene amplification and overexpression, as well as protein accumulation, can also occur in ductal carcinoma in situ (DCIS) (Weinstat-Saslow et al., 1995, Simpson et al., 1997, Alle et al., 1998, Zhu et al., 1998, Gillett et al., 1998) and breast cancer (Buckley et al., 1993, Zukerberg et al., 1995, Zhang et al.,

1994, Frierson et al., 1996, Barbareschi et al., 1997). In the latter, cyclin D1 accumulation, as detected immunohistochemically, has been observed in up to 81% of cases (Zukerberg et al., 1995) and gene amplification has been observed in between 11 and 23% of cases (Zhang et al., 1994, Frierson et al., 1996, Lammie et al., 1991, Worsley et al., 1996, Courjal et al., 1996). In the present study cyclin D1 gene amplification and protein overexpression were detected in breast tissues at similar frequencies to those reported by others. However, we did not observe associations between the presence of either or both of these cyclin D1 alterations and breast cancer risk. Furthermore, the presence of either cyclin D1 amplification and/or protein overexpression in combination with epithelial hyperplasia was not associated with altered risk. There were too few cases of epithelial hyperplasia with atypia to permit a statistically meaningful evaluation of risk in association with this histological abnormality and cyclin D1 changes.

The benign breast tissue of 75 subjects showed positive immunostaining whereas the benign tissue of only 41 subjects showed gene amplification. The lack of correlation between cyclin D1 gene amplification and protein overexpression has been described previously (Frierson et al., 1996, Simpson et al., 1997, Zhu et al., 1998, Worsley et al., 1996, Pelosio et al., 1996). It has been suggested that mechanisms other than gene amplification, such as post-transcriptional or post-translational mechanisms, could cause increased levels of cyclin D1 protein (Simpson et al., 1997, Worsley et al., 1996). For example, accumulation of cyclin D1 protein can occur because of transactivation of the cyclin D1 promoter by β-catenin (Lin et al., 2000), or increased stability of the protein, as has been demonstrated in human uterine sarcomas (Welcker et al., 1996), or decreased proteolysis, as has been shown to occur in the MCF-7 breast cancer cell line (Russell et al., 1999). The mechanism(s) causing cyclin D1 protein overexpression in the absence of gene amplification in these breast tissues is unknown.

It is possible that the methodology used in this study influenced the results. The differential PCR assay used to determine whether gene amplification was present was assessed previously for sensitivity and reproducibility (Zhu et al., 1998). Although a different housekeeping gene was used in that study, the results suggested that differential PCR is appropriate for determining whether the cyclin D1 gene is amplified and that it is sufficiently sensitive to detect two-fold gene amplification. In addition, other studies have utilized this approach for semiquantitation of gene amplification (Nakagawa et al., 1995, Suzuki et al., 1998, Schneeberger et al., 1998). In terms of the immunostaining, the methodology that was used may have resulted in an underestimation of the number of subjects overexpressing cyclin D1. It has been shown that the type and duration of tissue fixation, the sensitivity of the antibody, and the extent of tissue sampling may influence the sensitivity of immunohistochemistry (Rohan et al., 1998, Elias 1990). Also, given that the controls were women with biopsy proven benign breast disease and that their risk for subsequent breast cancer is higher than women without benign breast disease (Page DL and Anderson TJ, 1987) it is possible that we underestimated the magnitude of the association between cyclin D1 gene overexpression or protein accumulation and breast cancer risk.

Other than methodological limitations, there are several possible reasons why cyclin D1 changes were not associated with altered breast cancer risk. First, recent experimental data suggest that cyclin D1 is not a dominant oncogene but requires the presence of other oncogenes to form tumors (Barnes and Gillett, 1998). For example, transformation of BRK cells occurred when cyclin D1 was transfected together with the adenovirus E1A oncogene (Hinds et al., 1994) and transformation of rat fibroblasts by cyclin D1 required the presence of Ha-ras (Lovec et al., 1994). Also, transgenic mice containing cyclin D1 linked to an immunoglobulin enhancer rarely developed lymphoma until the mice were crossed with mice expressing the myc transgene

(Bodrug et al., 1994). Secondly, the quantification of cyclin D1 gene amplification suggested that the level of amplification in the benign tissue was in the range of that observed in the ZR-75-1 cell line, which shows two to five-fold amplification. This low level of amplification may be insufficient to affect cell proliferation. Thirdly, the effect of cyclin D1 on the cell cycle is controversial, as stable transfection of cyclin D1 into HBL-100, a mammary epithelial cell line, resulted in longer doubling time, an increased percentage of cells in S phase, and decreased tumorigenesis (Han et al., 1995). This is different from the effect observed for rat fibroblasts, suggesting that the effect of cyclin D1 overexpression may be dependent on the cell type and which other genes are expressed (Jiang et al., 1993). Fourthly, the presence of increased cyclin D1 mRNA levels (Utsumi et al., 2000) or moderate to strong staining for cyclin D1 (Gillett et al., 1996) in breast cancer has been associated with a better prognosis, an observation that raises the possibility that cyclin D1 may not be involved in the pathogenesis of breast cancer.

In conclusion, in this study cyclin D1 amplification and/or protein overexpression in normal or benign breast tissue were not associated with increased risk of developing breast cancer. As experimental evidence suggests that cyclin D1 requires other oncogenes to induce tumorigenesis, assessment of cyclin D1 alterations alone may not be sufficient to identify women at increased risk of breast cancer. Instead, it may not be until the cascade of molecular alterations leading to breast cancer development (Beckmann et al., 1997, Ingvarsson, 1999) are known that the putative role of cyclin D1 in this process can be identified.

FIGURE LEGENDS

Figure 1

Nuclear immunostaining for cyclin D1 (↑) in scattered benign epithelial cells (immunoperoxidase with hematoxylin counterstain, magnification x 640).

Figure 2

Ethidium bromide stained gel of cyclin D1 (**CD1**) and dopamine receptor (**DR**) from 15 subjects. For 2 subjects, corresponding stromal tissue (**S**), which did not contain any breast epithelium, was also analyzed as a control (ZR=ZR-75-1; MD=MDA-MB-231, 100bp = size ladder).

REFERENCES

Alle KM, Henshall SM, Field AS, Sutherland RL. (1998). Cyclin D1 protein is overexpressed in hyperplasia and intraductal carcinoma of the breast. *Clin. Cancer Res.*, 4, 847-854.

Barbareschi M, Pelosio P, Caffo O, Buttitta F, Pellegrini S, Barbazza R, Dalla Palma P, Bevilacqua G, Marchetti A. (1997). Cyclin D1-gene amplification and expression in breast carcinoma: relation with clinicopathologic characteristics and with retinoblastoma gene product, p53, and $p21^{WAF1}$ immunohistochemical expression. *Int J. Cancer*, 74, 171-174.

Barnes DM, Gillett CE. (1998). Cyclin D1 in breast cancer. Breast Cancer Res. Treat., 52, 1-15.

Bartkova J, Lukas J, Müller J, Lützhøft D, Strauss M, Bartek, J. (1994). Cyclin D1 protein expression and function in human breast cancer. *Int. J. Cancer*, 57, 353-361.

Beckmann MW, Niederacher D, Schnürch HG, Gusterson BA, Bender HG. (1997). Multistep carcinogenesis of breast cancer and tumour heterogeneity. *J. Mol. Med.*, 75, 429-439.

Bodrug SE, Warner BJ, Bath ML, Lindeman GJ, Harris AW, Adams JM. (1994). Cyclin D1 transgene impedes lymphocyte maturation and collaborates in lymphomagenesis with the myc gene. *EMBO J*, 13, 2124-2130.

Breslow NE, Day NE. Statistical methods in cancer research. Vol I, No. 32. (1980). Lyon: IARC Scientific Publications.

Buckley MF, Sweeney KJE, Hamilton JA, Sini RL, Manning DL, Nicholson RI, deFazio A, Watts CKW, Musgrove EA, Sutherland RL (1993). Expression and amplification of cyclin genes in human breast cancer. *Oncogene*, 8, 2127-2133.

Courjal F, Louason G, Speiser P, Katsaros D, Zeillinger R, Theillet, C. (1996). Cyclin gene amplification and overexpression in breast and ovarian cancers: evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. *Int. J. Cancer*, 69, 247-253.

Elias JM.(ed.) (1996). Immunohistopathology. A Practical Approach to Diagnosis. ASCP Press: Chicago.

Frierson HF Jr, Gaffey MJ, Zukerberg LR, Arnold A, Williams ME (1996). Immunohistochemical detection and gene amplification of cyclin D1 in mammary infiltrating ductal carcinoma. *Mod. Pathol.*, 9, 725-730.

Frye RA, Benz CC, Liu E (1989). Detection of amplified oncogenes by differential polymerase chain reaction. *Oncogene*, 4, 1153-1157.

Gillett C, Smith P, Gregory W, Richards M, Millis R, Peters G, Barnes D. (1996). Cyclin D1 and prognosis in human breast cancer. *Int. J. Cancer*, 69, 92-99.

Gillett CE, Lee AHS, Millis R.R., Barnes DM. (1998). Cyclin D1 and associated proteins in mammary ductal carcinoma in situ and atypical ductal hyperplasia. *J. Pathol.*, 184, 396-400.

Gramlich TL, Fritsch CR, Maurer D, Eberle M, Gansler TS. (1994). Differential polymerase chain reaction assay of cyclin D1 gene amplification in esophageal carcinoma. *Diagn. Mol. Pathol.*, 3, 255-259.

Grandy DK, Marchionni MA, Makam H, Stofko RE, Alfano M., Frothingham L, Fischer JB, Burke-Howie KJ, Bunzow JR, Server AC, Civelli O. (1989). Cloning of the cDNA and gene for a human D_2 dopamine receptor. *Proc. Natl. Acad. Sci. USA* , 86, 9762-9766.

Han, EK-H, Sgambato A, Jiang W, Zhang Y-J, Santella RM, Doki Y, Cacace AM, Schieren I, Weinstein IB. (1995). Stable overexpression of cyclin D1 in a human mammary epithelial cell line prolongs the S-phase and inhibits growth. *Oncogene*,10, 953-961.

Hinds PW, Dowdy SF, Eaton EN, Arnold A, Weinberg RA. (1994). Function of a human cyclin gene as an oncogene. *Proc. Natl. Acad. Sci. USA*, 91, 709-713.

Ingvarsson S. (1999). Molecular genetics of breast cancer progression. *Sem. Cancer Biol.*, 9, 277-288.

Jiang W, Kahn SM, Zhou P, Zhang Y-J, Cacace AM, Infante AS, Doi S, Santella RM, Weinstein IB. (1993). Overexpression of cyclin D1 in rat fibroblasts causes abnormalities in growth control, cell cycle progression and gene expression. *Oncogene*, 8, 3447-3457.

Lammie GA, Fantl, V, Smith R, Schuuring E, Brookes S, Michalides R, Dickson C, Arnold A, and Peters G. (1991). G. D11S287, a putative oncogene on chromosome 11q13, is amplified and expressed in squamous cell and mammary carcinomas and linked to BCL-1. *Oncogene*, 6, 439-444.

Lin S-Y, Xia W, Wang JC, Kwong KY, Spohn B. Wen Y, Pestell RG, Hung M-C. (2000). β-Catenin, a novel prognostic marker for breast cancer: Its roles in cyclin D1 expression and cancer progression. *Proc. Nat'l Acad. Sci.*, 97, 4262-4266.

Lovec H, Sewing A, Lucibelo FC, Muller R, Moroy T. (1994). Oncogenic activity of cyclin D1 revealed through cooperation with Ha-ras: link between cell cycle control and malignant transformation. *Oncogene*, 9, 323-326.

Miller AB, Baines CJ, To T, Wall C. (1992). Canadian National Breast Screening Study. I. Breast cancer detection and death rates among women aged 40 to 49 years. II. Breast cancer detection and death rates among women aged 50 to 59 years. *Can. Med. Assoc. J.*, 147, 1459-1488.

Miller AB, Howe GR, Wall C. (1981). The national study of breast cancer screening protocol for a Canadian randomized controlled trial of screening for breast cancer in women. *Clin. Invest. Med.*, 4, 227-258.

Nakagawa H, Zukerberg L, Togawa K, Meltzer SJ, Nishihara T, Rustgi AK. (1995). Human cyclin D1 oncogene and esophageal squamous cell carcinoma. *Cancer*, 76, 541-549.

Neubauer A, Neubauer B, He M, Effert P, Iglehart D, Frye RA, Liu E. (1992). Analysis of gene amplification in archival tissue by differential polymerase chain reaction. *Oncogene* 7, 1019-1025.

Pagano M, Theodoras AM, Tam SW, Draetta GF (1994). Cyclin D1-mediated inhibition of repair and replicative DNA synthesis in human fibroblasts. *Genes Dev.*, 8, 1627-1639.

Page DL, Anderson TJ. (eds.) (1987). Diagnostic Histopathology of the Breast. Churchill Livingstone: Edinburgh.

Pelosio P, Barbareschi M, Bonoldi E, Marchetti A, Verderio P, Caffo O. Bevilacqua P, Boracchi, P, Buttitta F, Barbazza R, Dalla Palma P, Gasparini G. (1996). Clinical significance of cyclin D1 expression in patients with node-positive breast carcinoma treated with adjuvant therapy. Ann. *Oncol.*, 7, 695-703.

Prall OW, Rogan EM, Sutherland RL (1998) Estrogen regulation of cell cycle progression in breast cancer cells. J Steroid Biochem Mol Biol., 65,169-174.

Rohan TE, Hartwick W, Miller AB, Kandel RA. (1998). Immunohistochemical detection of c-erbB-2 and p53 in benign breast disease and breast cancer risk. *J. Natl. Cancer Inst.*, 90, 1262-1269.

Russell A, Thompson MA, Hendley J, Trute L, Armes J, Germain D. (1999). Cyclin D1 and D3 associate with the SCF complex and are coordinately elevated in breast cancer. *Oncogene*, 18: 1983-1991.

Saddik M, Lai R, Medeiros LJ, McCourty A, Brynes,RK. (1999). Differential expression of cyclin D1 in breast papillary carcinomas and benign papillomas. *Arch. Pathol. Lab. Med.*, 123, 152-156.

Schneeberger C, Eder S, Swoboda H, Ullrich R, Zeillinger RA. (1998). Differential PCR system for the determination of CCND1 (cyclin D1) gene amplification in head and neck squamous cell carcinomas. *Oral Oncol.*, 34, 257-260.

Schwartz GF, Lagios, MD, Carter D, Connolly J, Ellis IO, Eusebi V, Finkel GC, Gorstein F, Holland R, Hutter RVP. (1997). Consensus conference on the classification of ductal carcinoma in situ. *Hum. Pathol.*, 28, 1221-1224.

Simpson JF, Quan DE, O'Malley F, Odom-Maryon T, Clarke PE. (1997). Amplification of CCND1 and expression of its protein product, cyclin D1, in ductal carcinoma in situ of the breast. *Am. J. Pathol.*, 151, 161-168.

Steeg PS, Zhou Q. (1998). Cyclins and breast cancer. *Breast Cancer Res. Treat.*, 52, 1728.

Suzuki H, Moriya J, Nakahata A, Fujioka Y, Inoue K, Nagashima K. (1998). Cyclin D1 gene amplification in esophageal carcinosarcoma shown by differential polymerase chain reaction. *Hum. Pathol.*, 29, 662-667.

Utsumi T, Yoshimura N, Maruta M, Takeuchi S, Ando J, Mizoguchi Y, Harada N. (2000). Correlation of cyclin D1 mRNA levels with clinico-pathological parameters and clinical outcome in human breast carcinomas. *Int. J. Cancer (Pred. Oncol.)*: 89, 39-43.

Weinstat-Saslow D, Merino MJ, Manrow RE, Lawrence JA, Bluth RF, Wittenbel KD, Simpson, JF, Page DL, Steeg PS. (1995). Overexpression of cyclin D mRNA distinguishes invasive and in situ breast carcinomas from non-malignant lesions. *Nat. Med.*, 1, 1257-1260.

Welcker M, Lukas J, Strauss M, Bartek J.(1996). Enhanced protein stability: a novel mechanism of D-type cyclin over-abundance identified in human sarcoma cells. *Oncogene*, 13, 419-425.

Worsley SD, Jennings BA, Khalil KH, Mole M, Girling AC. (1996). Cyclin D1 amplification and expression in human breast carcinoma: correlation with histological prognostic markers and oestrogen receptor expression. *J. Clin. Pathol. Mol. Pathol.*, 49, M46-M50.

Xiong Y, Zhang H, Beach D. (1992). D type cyclins associate with multiple protein kinases and the DNA replication and repair factor PCNA. *Cell*, 71, 505-514.

Zhang S-Y, Caamano J, Cooper F, Guo X, Klein-Szanto AJP. (1994). Immunohistochemistry of cyclin D1 in human breast cancer. *Am. J. Clin. Pathol.*, 102, 695-698.

Zhou P, Jiang W, Weghorst CM, Weinstein IB. (1996). Overexpression of cyclin D1 enhances gene amplification. *Cancer Res.*, 56, 36-39.

Zhou Q, Wulfkuhle J, Ouatas T, Fukushima P, Stetler-Stevenson M., Miller FR, Steeg, PS. (2000). Cyclin D1 overexpression in a model of human breast premalignancy: preferential stimulation of anchorage-independent but not anchorage-dependent growth is associated with increased cdk2 activity. *Breast Cancer Res. Treatment*, 59, 27-39.

Zhu XL, Hartwick W, Rohan T, Kandel R. (1998). Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. *Mod. Pathol.*, 11, 1082-1088.

Zukerberg LR, Yang W-I., Gadd M, Thor AD, Koerner FC, Schmidt EV, Arnold A. (1995). Cyclin D1 (PRAD1) protein expression in breast cancer: approximately one-third of infiltrating mammary carcinomas show overexpression of the cyclin D1 oncogene. *Mod. Pathol.*, 8, 560-567.

Table 1. Primer sequences and PCR conditions

Primers	Sequences	Product size(bp)	PCR Conditions
Cyclin D1	5'-ACCAGCTCCTGTGCTGCGAA-3'	152 bp	30 cycles, 95°C, 1 min, 55°C, 1 min, 72°C, 1 min
	5'-CAGGACCTCCTTCTGCACAC-3'		
Dopamine Receptor	5'-CCACTGAATCTGTCCTGGTATG-3'	113 bp	30 cycles, 95°C, 1 min, 55°C, 1 min, 72°C, 1 min
	5'-GCGTGGCATAGTAGTTGTAGTGG-3'		
□□IFN	5'-TCTTTTCTTTCCCGATAGGT-3'	150 bp	30 cycles, 95°C, 1 min, 50°C, 1 min, 72°C, 1 min
	5'-CTGGGATGCTCTTCGACCTC-3'		
□□IFN	5'-GCAGAGCCAAATTGTCTCCT-3'	82 bp	30 cycles, 95°C, 1 min, 50°C, 1 min, 72°C, 1 min
	5'-GGTCTCCACACTCTTTTGGA-3'		

Table 2. Association between cyclin D1 gene amplification or protein overexpression and risk of breast cancer

Aspect of staining	Aspect	Level	No. cases[a]	No. controls	Unadjusted OR (95%CI)[b]	Adjusted OR (95%CI)[c]
Immuno-staining	Presence	Absent	56	233	1[d]	1[d]
		Present	15	60	1.07(0.56-2.03)	1.06 (0.56-2.02)
	% cells immuno-positive	Absent	56	233	1[d]	1[d]
		<10	11	41	1.14 (0.55-2.38)	1.08 (0.49-2.37)
		>10	4	19	0.91 (0.29-2.89)	1.04 (0.31-3.48)
Gene amplification	Presence	Absent	57	258	1[d]	1[d]
		Present	12	29	1.67 (0.78-3.58)	1.41 (0.62-3.22)

[a]Unmatched distributions (matched odds ratios cannot be calculated directly from these numbers).
[b]Adjusted for matching factors only (using conditional logistic regression).
[c]Adjusted for hyperplasia, age at menarche, age at first livebirth, menopausal status, Quetelet's index, and history of breast cancer in a first degree relative. OR = odds ratio, CI = confidence interval.
[d] Reference category.

Table 3. Concordance between cyclin D1 immunostaining and gene amplification results for benign and malignant disease

Aspect	Percent negative on benign and cancer (No.)[a,b]	Percent negative on benign, positive on cancer (No.)	Percent positive on benign, negative on cancer (No.)	Percent positive on benign and cancer (No.)
Immuno-staining	50.9 (27)	22.6 (12)	7.5 (4)	18.9 (10)
Gene amplification	61.9 (26)	16.7 (7)	16.7 (7)	4.8 (2)

[a]Percentages are percent of all subjects for whom both benign and malignant tissue was available.

[b]Of the 62 cases for whom blocks of benign and malignant tissue were obtained, 2 blocks of benign tissue and 7 of blocks of malignant tissue were unsuitable for analysis. For the analysis of gene amplification, the tissue was inadequate for analysis for 7 cancers and no DNA was extracted from 4 cancers.

Correlation of p53 Mutations in ThinPrep® Processed Fine Needle Breast Aspirates with Surgically Resected Breast Cancers

Aaron Pollett, MD, Yvan C. Bédard, MD, Shu-Qiu Li,
Tom Rohan, MD, PhD, and Rita Kandel MD

Department of Pathology and Laboratory Medicine, Mount Sinai Hospital, Toronto, Ontario, Canada (AP,YCB,S-QL,RK)

Department of Epidemiology and Social Medicine, Albert Einstein College of Medicine, Bronx, New York (TR)

Send page proofs and correspondence to:
Dr. Rita Kandel
Department of Pathology and Laboratory Medicine
Mount Sinai Hospital. Room 600
600 University Avenue
Toronto. Ontario M5G 1X5
CANADA

Running title: p53 mutations in breast aspirates and breast cancer

ABSTRACT

Mutations of the p53 gene are one of the most common genetic changes found in cancer and their presence may be prognostic and even influence treatment for breast cancer. In this study we investigated whether DNA could be extracted from the residual cells left in ThinPrep® processed breast fine needle aspirates and whether p53 gene changes could be detected in the DNA. The results were then correlated with DNA extracted from the matched formalin-fixed, paraffin-embedded, surgically resected breast cancer when available. DNA was successfully extracted from 54 of 62 aspirates and all 31 surgical specimens. p53 gene mutations were detected in 10 of the 54 cytology specimens (18.5%) and consisted of base pair substitutions or deletions. Silent or intronic p53 changes were found in five additional aspirates. One of the aspirates had 2 gene alterations resulting in a total of 6 gene changes. Five of these changes were located in introns 6 or 9 and the sixth was a silent (no amino acid change) change in exon 6. p53 polymorphisms were detected in 9 aspirates (16.3%) and were located in codon 47 (one aspirate), codon 72 (six aspirates) and codon 213 (two aspirates). All cases with surgical material available showed identical p53 mutations, alterations and polymorphisms in the resected tumors when compared to those detected in the corresponding aspirates. The results of this study show that DNA suitable for analysis of p53 gene sequence changes can be successfully extracted from ThinPrep® processed breast FNAs, and that identical alterations are detected in both the cytology and surgical specimens.

KEY WORDS: ThinPrep®, fine needle aspiration, breast cancer, p53 mutation

INTRODUCTION

Mutations of the p53 gene are amongst the most common molecular changes detected in human cancers (1). Experimental studies have shown that functional p53 is required for the in vitro cytotoxic action of some chemotherapeutic agents (2) The presence of p53 mutations is associated with an increased chemoresistance to doxyrubicin in breast cancer patients (3) and may be involved in the development of multidrug resistance (4). Clinical studies have shown that breast cancers that contain p53 gene mutations are associated with decreased disease-free and overall survival (3,5-9). These results suggest that the presence of p53 mutations might provide prognostic information and influence the treatment of the breast cancer.

Fine-needle aspiration (FNA) of the breast is a safe, effective method for diagnosing breast cancer with minimal intervention and complications (10,11). As reviewed by Bédard et al., for the detection of carcinoma, it has a sensitivity ranging from 74% to 97% and a specificity ranging from 82% to 100% (12). ThinPrep® processed and conventionally processed breast FNA have been shown to have similar diagnostic accuracy (12). In addition, immunohistochemistry (13,14) and molecular analysis (15-17) have been successfully applied to ThinPrep® processed specimens.

As FNA is often the initial sampling of the tumor, it could be a source of cells for the early detection of p53 mutations. In this study, we examined whether p53 mutations could be detected in the cells present in the residual fluid from ThinPrep© processed breast FNAs. When available the corresponding paraffin-embedded surgically resected tissue was also analyzed for p53 mutations and the results were correlated.

MATERIALS AND METHODS

Specimen Acquisition, Clinical History and Pathology Review:

Cytology reports from November 1997 to April 1999 in the files of Mount Sinai Hospital were reviewed. Of the cases diagnosed as positive or suspicious for malignancy, DNA could be extracted from 54 of 62 specimens of ThinPrep® processed breast FNA obtained from 62 different women. In cases where DNA was successfully extracted from the cytology fluid the surgical pathology records were reviewed to determine whether there was a corresponding breast tumor specimen. Formalin fixed paraffin-embedded tissue was available for 31 women. Clinical details and tumor characteristics were obtained from surgical reports. The breast cancers were graded according to the Elston's Modified Bloom and Richardson criteria (18). In 30 of the 31 surgical specimens, the tumor was removed after the cytology specimen. On average the specimen was removed 33 days after the FNA (range 8 – 72 days). In one case the FNA was from a tumor recurrence in the scar 6 weeks after the mastectomy.

p53 Molecular Analysis:

DNA Extraction

Cytology: After completing the cytological examination the residual preservative fluid (PreservCyt® solution, Cytyc Corporation, Boxborough, MA.) was stored at 4°C for up to 3 months. The fluid was centrifuged at 4000 g and the supernatant removed. DNA was extracted from the remaining cells using TriZol® (GibcoBRL, Rockville, MD). DNA extraction was performed according to the manufacturer's instructions for cells grown in suspension. The DNA was stored at 4°C until used for analysis.

Surgical Specimens: Five µm sections were cut from the paraffin blocks and stored for up to 2 weeks. Prior to microdissection the sections were dewaxed and stained briefly with hematoxylin.

A representative portion of the tumor containing minimal numbers of stromal and inflammatory cells was microdissected and placed in a microfuge tube. The tissue was digested with proteinase K (0.5 mg/ml in 50 mM TrisHCl, pH 8.5, 10 mM EDTA, 0.5% Tween 20) for at least 48 hours at 55°C (19). The proteinase K was inactivated by heating at 95°C for 15 minutes. The DNA was stored at -20°C for up to 3 wks until further analyzed.

Polymerase Chain Reaction- Single Strand Conformational Polymorphism Analysis:

A 1ul aliquot from each sample was added to 14 ul of PCR solution containing 1.5 mM $CaCl_2$, 20 mM Tris HCl (pH 8.0), 50 mM KCl, 0.25 uM of each primer, 0.1 mM of each dNTP, 1U Taq DNA polymerase (GibcoBRL, Rockville, MD), and 2 uCi [α-^{33}P]-dATP. The primers and the cycling conditions for each exon are listed in Table 1. The reaction product was run on an 8% non-denaturing polyacrylamide gel and the gel was processed for autoradiography (20,21). Potential mutations were detected by shifts in band mobility. If there was no band shift, the tissue was considered to have no mutation. For samples showing band shifts, the PCR-SSCP analysis was repeated. In cases where different band shifts were detected in the cytology and corresponding paraffin-embedded samples an additional paraffin block was selected, cut, microdissected, and processed as above. Negative controls, paraffin-embedded cells that contained no p53 mutation in the exon examined and a water control to replace the DNA, were included in each analysis. Positive controls for exons 5 to 9 (exon 5: SKBr3; exon6: T-47D; exon 7: colo 320DM; exon 8: MDA-MB-468; exon 9: SW480) were also included where appropriate.

p53 sequencing: The abnormally shifted band was excised from the SSCP gel and the DNA was eluted into water. The DNA was reamplified by PCR using the same primers and the product was run on a 2% agarose gel. The band was extracted using a QIAquick Gel Extraction Kit (QIAGEN, Chatsworth, CA). The purified DNA was sequenced using a ThermoSequenase

radiolabelled terminator cycle sequencing kit (Amersham Life Sciences. Cleveland. Ohio) and the sense primer according to the manufacturer's directions. followed by gel electrophoresis and autoradiography. To confirm the mutation, the DNA product was resequenced using the antisense primer. Negative controls were included in each analysis. Cell lines with known mutations in exons 5 to 9 were also included where appropriate. Mutations were compared to those mutations listed for breast cancer in a known p53 database (www.iarc.fr p53) (22).

Statistical Analysis:

The associations between p53 gene alterations and clinical tumor variables were examined using the Chi-square (χ^2) or, where appropriate. Fisher's exact test (23). Two-sided p-values below 0.05 were considered to be statistically significant.

RESULTS

Histological review of the 31 surgically resected breast tumors showed that they consisted of 29 infiltrating ductal carcinomas not otherwise specified. 1 invasive ductal carcinoma with lobular features. and 1 mucinous carcinoma. DNA was successfully extracted from all paraffin-embedded tumors.

Of 62 cytology samples. DNA suitable for p53 sequencing was extracted from 54. yielding an evaluable specimen in 87% of the cases. p53 gene mutations were detected in ten of the 54 cytology specimens (18.5%). As shown in table 2. these consisted of base pair substitutions and deletions. For 8 of these 10 aspirates. surgically resected breast tumor tissue was available for gene analysis. All 8 cases showed identical p53 mutations in both the aspirate and the surgically resected tumor. A representative SSCP gel is shown in Figure 1 and the associated sequencing gel is shown in Figure 1B.

Other types of p53 gene changes were found in five other aspirates. One aspirate had 2 gene alterations resulting in a total of six gene changes. As shown in table 3, five changes were located in introns 6 or 9 and one was a silent change (no amino acid change) in exon 6. For two of these 5 aspirates, surgically resected breast tumor tissue was available for gene analysis and both of the cases showed identical p53 gene changes in the aspirate and the surgically resected tumor.

p53 polymorphisms were detected in nine aspirates (16.3%) and as shown in table 4 were located in codon 47 (one aspirate), codon 72 (six aspirates) and codon 213 (two aspirates). For seven of these 9 aspirates, surgically resected breast tumor tissue was available for gene analysis and all seven cases showed identical p53 polymorphisms in both the aspirate and the surgically resected tumor.

The clinical features and tumor characteristics were correlated with the p53 gene status and are summarized in Table 5. DNA suitable for p53 sequencing could be obtained from aspirates of tumors of all three grades. The women whose tumors had a p53 mutation or an intronic change or a silent change were grouped together for these analyses because of the small numbers. There was a significant correlation between a younger age ($p=0.038$) or larger tumor size ($p=0.046$) with the presence of p53 gene alterations. There was no correlation between the presence of estrogen ($p=0.449$) or progesterone ($p=0.066$) receptors or tumor grade ($p=0.227$) and the presence of p53 gene alterations.

DISCUSSION

This study demonstrated that DNA can be extracted from ThinPrep® processed breast FNAs. This is in keeping with the findings of other groups that have reported successful extraction of RNA or DNA from ThinPrep® processed cytology specimens of breast and cervix

(15-17). In addition, the current study showed that the extracted DNA was suitable for p53 gene analysis by PCR-SSCP and sequencing. Using the protocol described above, the mutations detected in exons 4 to 9 were identical to those found in the formalin fixed paraffin-embedded surgically resected breast cancer when this tissue was available for analysis. In contrast, studies assessing p53 immunoreactivity in FNAs and formalin fixed paraffin-embedded tumors have shown variable correlations ranging from 73.5% - 93.3% (24-26).

Recent studies have shown that gene alterations detected in paraffin-embedded tissue may be artifacts induced by fixation or processing of surgical specimens (27,28). Several precautionary steps were undertaken to minimize this possibility. The fidelity of the PCR amplification of DNA extracted from paraffin can be markedly improved by prolonged proteinase K digestion and using small DNA templates (29), so in this study the paraffin extracted DNA was digested by proteinase K for at least 48 hours and the primers were chosen to provide gene sequences of less than 300 base pairs in length. To ensure that the gene alterations were not due to nucleotide substitutions as a result of Taq DNA polymerase misincorporation, all specimens that had an abnormal SSCP underwent repeat PCR-SSCP to confirm that the change was reproducible. Only those samples that showed similar changes on the repeat PCR-SSCP were considered to have a sequence alteration, which was then confirmed by sequencing. Furthermore, identical alterations were seen in the methanol fixed aspirates and in the corresponding formalin fixed paraffin embedded surgically resected tumors. This would suggest that the p53 alterations identified in this study were genuine.

p53 mutations were found in 18.5% of patients. This is within the frequency reported for breast carcinoma in other series (8,9,30-34). The majority of changes reported for breast cancer have been point mutations (22) and in our series eight of the ten mutations (80%) involved base

pair substitutions. All mutations, except two (cytology specimens #7 and #13) have been previously reported to occur in breast cancer as listed in a p53 database (22). Silent gene changes were detected in 1.9% of patients and this is similar to the frequency (1.8%) reported by Burns et al. (6). In the database examined, there was no report of the silent change observed at codon 224 (cytology specimen #56). A similar comparison could not be done for the intronic alterations because the nucleotide position of these types of gene changes is not provided in the database. Codon 47 in exon 4, codon 72 in exon 4 and codon 213 in exon 6 contained known polymorphisms in one, six, and two patients respectively (1.8%, 11.1% and 3.7% of the patients). This is within the range determined for the normal population (35-37). As the frequencies of mutations and polymorphisms are similar to those shown by others, this suggests that our methodology to detect p53 gene changes is appropriate.

The presence of p53 alterations showed statistically significant associations with larger tumors and younger patient age. No significant association was seen between p53 alterations and tumor grade or the presence or absence of estrogen and progesterone receptors. Other studies examining the association between these clinical variables and p53 protein accumulation and/or mutations have yielded inconsistent and often conflicting results. For example, Caleffi et al. found that p53 mutations occurred in younger patients (38) but other studies have not found an association between age and p53 status (5,39,40). The number of patients in the current report is small and may have compromised the statistical power of the study to detect associations.

The use of residual cells from ThinPrep® processed samples has several advantages. Firstly the fluid from ThinPrep® processing can be stored at 4°C for up to three months, before extracting the DNA, as observed in the present study. Secondly, as only the residual fluid is needed for analysis, the original diagnostic slides do not have to be used. Thirdly, in contrast to

paraffin-embedded tissue which has to undergo proteinase K digestion for at least 48 hours before DNA extraction. ThinPrep® processed aspirates can undergo DNA extraction the same day it is obtained. However, there may also be disadvantages to using the residual material from ThinPrep® processing. Not all cases have tumor cells remaining in the residual fluid and thus DNA may not be available for analysis. In addition, if the aspirate contains numerous benign cells admixed with the malignant cells mutations may be missed (20,21).

Immunohistochemical staining can be used to detect p53 protein accumulation in either cytological or surgical specimens (24-26) but the immunohistochemical results do not always reflect the presence of underlying genetic changes (33,34,41,42). For example, nonsense mutations will not cause protein accumulation so these cells will be negative by immunohistochemical staining. In keeping with this, the presence of p53 mutations in the breast cancer was shown to be associated with decreased disease free survival as well as overall survival (5-9,31), but the presence of p53 protein detected immunohistochemically in the tumor has not consistently been associated with a worse prognosis (7,8,42). As molecular analysis of p53 may provide prognostic and treatment information for patients with breast cancer, ThinPrep aspirate is a suitable alternative to the paraffin-embedded tissue as a source of cells for this type of analysis in patients who will receive neoadjuvant chemotherapy or have unresectable tumours.

In summary, ThinPrep© processed breast fine needle aspirations provide DNA suitable for molecular analysis more rapidly than paraffin-embedded tissue. FNAs appear to be a reliable source of cells to determine the p53 gene status, given that identical alterations were detected in both the cytology and surgical specimens examined in this study.

Acknowledgements:

We thank Lori Cutler for her secretarial help. This work was funded in part by a grant from the US Army Medical Research and Materiel Command.

REFERENCES

1. Levine AJ, Momand J, Finlay CA. The p53 tumour suppressor gene. Nature 1991;351:453-6.
2. Lowe SW, Ruley HE, Jacks T, Housman DE. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell 1993;74:957-67.
3. Ogretmen B, Safa AR. Expression of the mutated p53 tumor suppressor protein and its molecular and biochemical characterization in multidrug resistant MCF- 7/Adr human breast cancer cells. Oncogene 1997;14:499-506.
4. Aas T, Borresen AL, Geisler S, Smith-Sorensen B, Johnsen H, Varhaug JE, Akslen LA, Lonning PE. Specific P53 mutations are associated with de novo resistance to doxorubicin in breast cancer patients. Nat Med 1996;2:811-14.
5. Berns EMJJ, Klijn JG, Smid M, van Staveren IL, Look MP, van Putten WLJ, Foekens JA. TP53 and MYC gene alterations independently predict poor prognosis in breast cancer patients. Genes Chromosomes Cancer 1996;16:170-79.
6. Berns EMJJ, van Staveren IL, Look MP, Smid M, Klijn JG, Foekens JA. Mutations in residues of TP53 that directly contact DNA predict poor outcome in human primary breast cancer. Br J Cancer 1998;77:1130-36.
7. Sjogren S, Inganas M, Norberg T, Lindgren A, Nordgren H, Holmberg L, Bergh J. The p53 gene in breast cancer: prognostic value of complementary DNA sequencing versus immunohistochemistry. J Natl Cancer Inst 1996; 88:173-82.
8. Elledge RM, Allred DC. Prognostic and predictive value of p53 and p21 in breast cancer. Breast Cancer Res Treat 1998;52:79-98.
9. Pharoah PD, Day NE, Caldas C. Somatic mutations in the p53 gene and prognosis in breast cancer: a meta-analysis. Br J Cancer 1999;80:1968-73.

10. The uniform approach to breast fine-needle aspiration biopsy. National Cancer Institute Fine-Needle Aspiration of Breast Workshop Subcommittees. Diagn Cytopathol 1997:16:295-311.

11. Wilkinson EJ, Bland KI. Techniques and results of aspiration cytology for diagnosis of benign and malignant diseases of the breast. Surg Clin North Am 1990:70:801-13.

12. Bedard YC, Pollett AF. Breast fine-needle aspiration. A comparison of ThinPrep® and conventional smears. Am J Clin Pathol 1999;111:523-27.

13. Leung SW, Bedard YC. Estrogen and progesterone receptor contents in ThinPrep-processed fine-needle aspirates of breast. Am J Clin Pathol 1999:112:50-56.

14. Leung SW, Bedard YC. Immunocytochemical staining on ThinPrep processed smears. Mod Pathol 1996:9:304-06.

15. Magda JL, Minger BA, Rimm DL. Polymerase chain reaction-based detection of clonality as a non-morphologic diagnostic tool for fine-needle aspiration of the breast. Cancer 1998: 84:262-67.

16. Dimulescu I, Unger ER, Lee DR, Reeves WC, Vernon SD. Characterization of RNA in cytologic samples preserved in a methanol-based collection solution. Mol Diagn 1998:3:67-71.

17. Sherman ME, Schiffman MH, Lorincz AT, Herrero R, Hutchinson ML, Bratti C, Zahniser D, Morales J, Hildesheim A, Helgesen K, Kelly D, Alfaro M, Mena F, Balmaceda I, Mango L., Greenberg M. Cervical specimens collected in liquid buffer are suitable for both cytologic screening and ancillary human papillomavirus testing. Cancer 1997:81:89-97.

18. Elston CW, Ellis IO. Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 1991:19:403-10.

19. Zhu XL, Hartwick W, Rohan T, Kandel R. Cyclin D1 gene amplification and protein expression in benign breast disease and breast carcinoma. Mod Pathol 1998;11:1082-88.

20. Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 1989;5:874-21.

21. Murakami Y, Hayashi K, Sekiya T. Detection of aberrations of the p53 alleles and the gene transcript in human tumor cell lines by single-strand conformation polymorphism analysis. Cancer Res 1991;51:3356-61.

22. Hainaut P, Hernandez T, Robinson A, Rodriguez-Tome P, Flores T, Hollstein M, Harris CC, Montesano R. IARC Database of p53 gene mutations in human tumors and cell lines: updated compilation, revised formats and new visualisation tools. Nucleic Acids Res 1998;26:205-13.

23. Dawson-Saunders B, Trapp RG. Basic and Clinical Biostatistics. Norwalk, Connecticut: Appleton and Lange, 1994.

24. Tiniakos DG, Robinson KB, Greenwood H, Cullen P, Cook AIM, Horne CHW, Angus B. c-erb B-2 and p53 expression in breast cancer fine needle aspirates. Cytopathology 1996;7:178-86.

25. Colecchia M, Frigo B, Zucchi A, Leopardi O. p53 protein expression in fine-needle aspirates of breast cancer: an immunocytochemical assay for identifying high-grade ductal carcinomas. Diagn Cytopathol 1995;13:128-32; discussion 132-33.

26. Makris A, Allred DC, Powles TJ, Dowsett M, Fernando IN, Trott PA, Ashley SE, Ormerod MG, Titley JC, Osborne CK. Cytological evaluation of biological prognostic markers from primary breast carcinomas. Breast Cancer Res Treat 1997;44:65-74.

27. Williams C, Ponten F, Moberg C, Soderkvist P, Uhlen M, Ponten J, Sitbon G, Lundeberg J. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155:1467-71.

28. Wong C, DiCioccio RA, Allen HJ, Werness BA, River MS. Mutations in BRCA1 from fixed, paraffin-embedded tissue can be artifacts of preservation. Cancer Genet Cytogenet 1998; 107:21-27.

29. Shiao YH, Buzard GS, Weghorst CM, Rice JM. DNA template as a source of artifact in the detection of p53 gene mutations using archived tissue. Biotechniques 1997;22:608-10, 612.

30. Dunn JM, Hastrich DJ, Newcomb P, Webb JC, Maitland NJ, Farndon JR. Correlation between p53 mutations and antibody staining in breast carcinoma. Br J Surg 1993; 80:1410-12.

31. Saitoh S, Cunningham J, De Vries EM, McGovern RM, Schroeder JJ, Hartmann A, Blaszyk H, Wold LE, Schaid D, Sommer SS et al. p53 gene mutations in breast cancers in midwestern US women: null as well as missense-type mutations are associated with poor prognosis. Oncogene 1994;9:2869-75.

32. Osborne RJ, Merlo GR, Mitsudomi T, Venesio T, Liscia DS, Cappa AP, Chiba I, Takahashi T, Nau MM, Callahan R et al. Mutations in the p53 gene in primary human breast cancers. Cancer Res 1991;51:6194-98.

33. Sato T, Yuyama Y, Watabe K, Okazaki A, Toda K, Okazaki M, Hirata K. Detection of p53 gene mutations in fine-needle aspiration biopsied breast cancer specimens: correlations with nuclear p53 accumulation and tumor DNA aneuploidy patterns. Cancer Lett 1997;115:47-55.

34. Lavarino C, Corletto V, Mezzelani A, Della Torre G, Bartolli C, Riva C, Pierotti MA, Rilke F, Pilotti S. Detection of TP53 mutation, loss of heterozygosity and DNA content in fine-needle aspirates of breast carcinoma. Br J Cancer 1998;77:125-30.

35. Felley-Bosco E, Weston A, Cawley HM, Bennett WP, Harris CC. Functional studies of a germ-line polymorphism at codon 47 within the p53 gene. Am J Hum Genet 1993;53:752-59.

36. Sjalander A, Birgander R, Hallmans G, Cajander S, Lenner P, Athlin L, Beckman G, Beckman L. p53 polymorphisms and haplotypes in breast cancer. Carcinogenesis 1996;17:1313-16.

37. Carbone D, Chiba I, Mitsudomi T. Polymorphism at codon 213 within the p53 gene. Oncogene 1991;6:1691-92.

38. Caleffi M, Teague MW, Jensen RA, Vnencak-Jones CL, Dupont WD, Parl FF. p53 gene mutations and steroid receptor status in breast cancer. Clinicopathologic correlations and prognostic assessment. Cancer 1994;73:2147-56.

39. Soong R, Iacopetta BJ, Harvey JM, Sterrett GF, Dawkins HJ, Hahnel R, Robbins PD. Detection of p53 gene mutation by rapid PCR-SSCP and its association with poor survival in breast cancer. Int J Cancer 1997;74:642-47.

40. Pelosi G, Bresaola E, Rodella S, Manfrin E, Piubello Q, Schiavon I, Iannucci A. Expressionof proliferating cell nuclear antigen, Ki-67 antigen, estrogen receptor protein, and tumor suppressor p53 gene in cytologic samples of breast cancer: an immunochemical study with clinical, pathobiological, and histologic correlations. Diagn Cytopathol 1994;11:131-40.

41. MacGeoch C, Barnes DM, Newton JA, Mohammed S, Hodgson SV, Ng M, Bishop DT, Spurr NK. p53 protein detected by immunohistochemical staining is not always mutant. Dis Markers 1993;11:239-50.

42. Jacquemier J, Moles JP, Penault-Llorca F, et al. p53 immunohistochemical analysis in breast cancer with four monoclonal antibodies: comparison of staining and PCR-SSCP results. Br J Cancer 1994:69:846-52.

TABLE 1. p53 PCR Primers and Cycling Conditions

Exon	Primer-sense (5`-3`) -antisense (5`-3`)	Product Size (bp)	Cycling Parameters
4	ATCTACAGTCCCCCTTGCCG GCAACTGACCGTGCAAGTCA	296	30 cycles: 50 sec at 95°C, 50 sec at 55°C, 60 sec at 72°C
5	GCTGCCGTGTTCCAGTTGCT CCAGCCCTGTCGTCTCTCCA	294	30 cycles: 50 sec at 95°C, 50 sec at 58°C, 60 sec at 72°C
6	GGCCTCTGATTCCTCAGTGA GCCACTGACAACCACCCTTA	199	30 cycles: 50 sec at 95°C, 50 sec at 55°C, 60 sec at 72°C
7	TGCCACAGGTCTCCCCAAGG AGTGTGCAGGGTGGCAAGTG	196	30 cycles: 50 sec at 95°C, 50 sec at 56°C, 60 sec at 72°C
8	CCTTACTGCCTCTTGCTTCT ATAACTGCACCCTTGGTCTC	225	30 cycles: 50 sec at 95°C, 50 sec at 55°C, 60 sec at 72°C
9	GCCTCAGATTCACTTTTATCACC CTTTCCACTTGATAAGAGGTCCC	152	30 cycles: 50 sec at 95°C, 50 sec at 56°C, 60 sec at 72°C

TABLE 2. Summary of p53 Mutations

Case Number				Sequence	Amino-Acid
Surgical	Cytology	Exon	Codon	Change	Change
20	13	5	*	del 23 bases	
9	7	5	130	C→T	Leu→Phe
10	3	5	175	G→A	Arg→His
36	61	5	183	C→G	Ser→STOP
38	29	6	209	del 2 bases	
13	19	6	220	A→C	Tyr→Ser
17	38	7	232	T→G	Ile→Ser
34	60	7	248	G→A	Arg→Gln
NA	59	8	270	T→C	Phe→Leu
NA	52	9	331	C→T	Gln→STOP

*deletion (del) starting at nucleotide residue 13041 in intron 4 and involving codons in exon 5.

NA = Tissue not available

TABLE 3. Summary of p53 Silent and Intronic Changes

Case Number				Sequence	Amino-Acid
Surgical	Cytology	Location	Site	Change	Change
NA	56	Exon 6	Codon 224	G→A	Glu→Glu
NA	18	Intron 6	nr* 13449	G→C	
NA	55	Intron 6	nr 13964	Del 1 base	
8	35	Intron 6	nr 13964	Del 1 base	
8	35	Intron 9	nr 14755	G→T	
15	5	Intron 9	nr 14766	T→C	

*nr=nucleotide residue

NA = Tissue not available

TABLE 4. Summary of p53 polymorphisms

Case Number				Sequence	Amino-Acid
Surgical	Cytology	Exon	Codon	Change	Change
2	36	Exon 4	47	C→T	Pro→Ser
15	5	Exon 4	72	G→C	Arg→Pro
NA	18	Exon 4	72	G→C	Arg→Pro
38	29	Exon 4	72	G→C	Arg→Pro
4	33	Exon 4	72	G→C	Arg→Pro
2	36	Exon 4	72	G→C	Arg→Pro
34	60	Exon 4	72	G→C	Arg→Pro
NA	37	Exon 6	213	A→G	Arg→Arg
31	39	Exon 6	213	A→G	Arg→Arg

NA = Tissue not available

TABLE 5. Patient and Tumor Features

Features	p53 Status		p-value
	Wild Type	Altered[a]	
Age			
<40	4	2	
40-55	3	7	**0.038**
56-70	6	1	
>70	7	1	
Tumor Size			
≤2 cm	6	3	
2-5cm	14	5	**0.046**
>5cm	0	3	
ER			
+	13	5	0.449
-	7	6	
PR			
+	13	3	0.066
-	7	8	
Grade			
1	3	0	
2	8	3	0.227
3	9	8	

[a]Altered p53 status includes mutations, silent and intronic changes for surgically resected tumors.

FIGURE LEGEND

Figure 1

A) A representative SSCP gel of p53 exon 7 PCR product from 3 cases and a negative control (Control). S-16 (surgically resected breast cancer) shows no abnormality. The cytology sample (C-38) and the corresponding paraffin-embedded surgical sample (S-17) show similar band shifts (→). B) The sequencing gel for samples C-38 and S-17 shows a T→G base substitution (→). The wild type sequencing pattern (control) in the same region is also shown.